SYNTHESIS AND APPLICATIONS OF BHC-DIOL: A NEW PHOTOREMOVABLE

PROTECTING GROUP

by

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(Under the Direction of Timothy M. Dore)

ABSTRACT

Photoremovable protecting groups have been used to study cell physiology. When covalently linked to biologically active molecules, they inactivate or "cage" these messengers. Reactivation or "uncaging" of physiological activity can be achieved with a flash of light. By multiphoton photolysis, the release of the messenger can be controlled in time, location, and amplitude. Caged compounds could be used in drug delivery. Because of a lack of physiologically useful caging groups for ketones and aldehydes, functional groups that are found in many biologically active molecules, such as steroids, a new photolabile protecting group based on a coumarin derivative, 6-bromo-4-(1,2-dihydroxyethyl)-7-hydroxycoumarin (Bhc-diol), was synthesized, and its photochemistry analyzed. Bhc-diol possesses sufficient one-photon quantum efficiency and two-photon uncaging cross-section for biological use. When conjugated to mifepristone, Bhc-diol has the promise to be a good photo-mediator of gene expression. Bhc-diol-mifepristone has been synthesized and its photochemical properties tested.

INDEX WORDS: caged compounds, Bhc-diol, photoremovable protecting groups, coumarin derivatives, multiphoton photolysis

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B. S., Jilin University, China, 1999

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment

of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2004

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May 2004

ACKNOWLEDGEMENTS

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CHAPTER 1

INTRODUCTION

PART 1. CAGED COMPOUNDS

Caged compounds are biologically inactive molecules that can liberate bioactive molecules of interest by a flash of light. The popular term "caged" refers to molecules whose biological recognition or activity has been disabled by chemical modification, especially by covalently linking them to photoremovable protecting groups (Figure 1). This term was first used in 1978 to describe photo-releaseable derivatives of natural molecules such as adenosine triphosphate (ATP) (Figure 2).[1] Covalent bond formation masks some important features for biological recognition and photochemical cleavage of the bond releases the bioactive compound. The rapid release of the bioactive molecule causes a sharp local increase in its concentration.

Figure 1. How Caged Compounds Work.

<div align="center">
caged
compound
(inactive messenger)   caging
                              remnant   active
                                               messenger
</div>
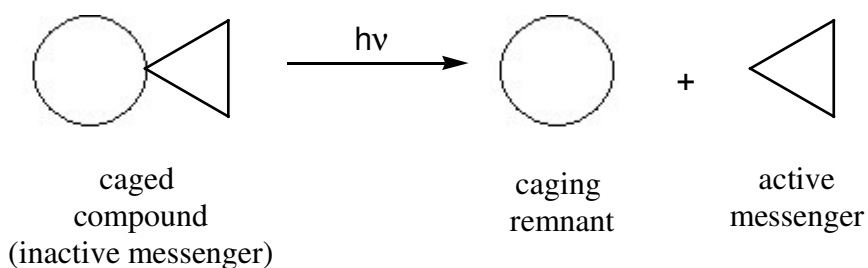
The key component of a "caged" compound is the photoremovable protecting group. Photoremovable protecting groups for various functional groups are useful in organic

synthesis,[2,3] biotechnology, drug discovery,[4-10] and cell biology[10,11] because they provide a different approach to the protection/deprotection process. Because photochemical reactions can be conducted under quite mild reaction conditions that are usually orthogonal to other experimental manipulations, photolysis is a less-damaging deprotection process even for relatively unstable molecules. The use of suitable photoremovable protecting groups has enabled caged compounds to become important tools to investigate and control the function of biological systems.

Figure 2. P[3]-1-(2-nitro)phenylethyl-adenosine Triphosphate (caged ATP)
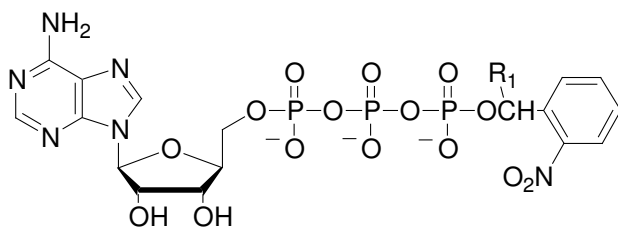
The effectiveness of a photoremovable protecting group in a biological system is judged by how well it satisfies the well-established criteria for common protecting groups[12,13] and how compatible it is with biological systems.[10] The necessary properties of a successful photoremovable protecting group for common biological substrates, such as ATP, L-glutamate, or γ-aminobutyric acid (GABA) are as follows: (1) The caged compound should not affect the biological system being studied; it cannot excite the biomolecule's normal stimulus before photolysis in the biological system. Any photoproducts other than the desired biomolecule should not interact or interfere with the biological system; (2) The photoproducts should not absorb at the same wavelength as the caged compounds, to avoid a light filtering effect by the product; (3) It should release the biomolecule rapidly in high yield and at wavelengths of light

that are not detrimental to the biological systems. The one-photon quantum efficiency of the photochemistry should be > 0.01 and the activating radiation should be more than 300 nm to avoid competition for the light by native biological chromophores; (4) The caged compound must be hydrolytically stable in high ionic aqueous buffer media in a dark environment; (5) The caged compound must be soluble in aqueous buffer. It is hard to find a photoremovable protecting group that can match all criteria; all five criteria do serve as important guidelines for the design and development of new photoremovable protecting groups.

Caged compounds are useful in biological investigation because the release of the messenger can be controlled in time, location, and amplitude.[11] By changing the concentration of bioactive molecules in a specific volume, the desired biological effect can be achieved without physically disturbing the system, especially inside an intact cell, tissue, or protein crystal. This technique is also very useful when microscopic spatial gradients are desired. Using caged compounds to control cell chemistry has become one of the best methods to study biology and biochemistry.[14-23]

Caged compounds generate active biomolecules within nanoseconds to milliseconds by absorbing photons. Light-directed activation of caged compounds, and rapid monitoring of the ensuing reaction using photomultiplier or imaging-based techniques have been used to understand the molecular mechanism of several biochemical reactions and processes in vitro and in vivo.[11,21,24,25] Caged compounds have also been used to study the molecular basis of neurotransmission, muscle contraction and ion channel function.[10] Many caged compounds have been created for these purposes, and there have been continuous efforts to exploit new photolabile protecting groups, such as benzoin-type (**3**),[26-30] phenacyl-type (**4**),[31,32] coumarinylmethyl-type (**5**),[33-36] and anthraquinon-2-ylmethoxycarbonyl,[1,36-41] since 2-

nitrobenzyl groups (**1**) were introduced to make caged derivatives of biologically important phosphates.[1,37] The major characters of common photoremovable protecting groups are summarized in Figure 3.

Figure 3. Common Photoremovable Protecting Groups

1: *o*-Nitrobenzyl alcohol derivatives

X= NR^{1}R^2, R, OR

2: Benzyl alcohol derivatives

R= H, NO$_2$, Br, MeO
X= O, N

3: Benzoin esters

X= OAc, NR$_2$

4: Phenacyl esters

5: Coumarin derivatives

R= H, Me   R'= H, Br
X= O, N

6: Quinoline derivatives
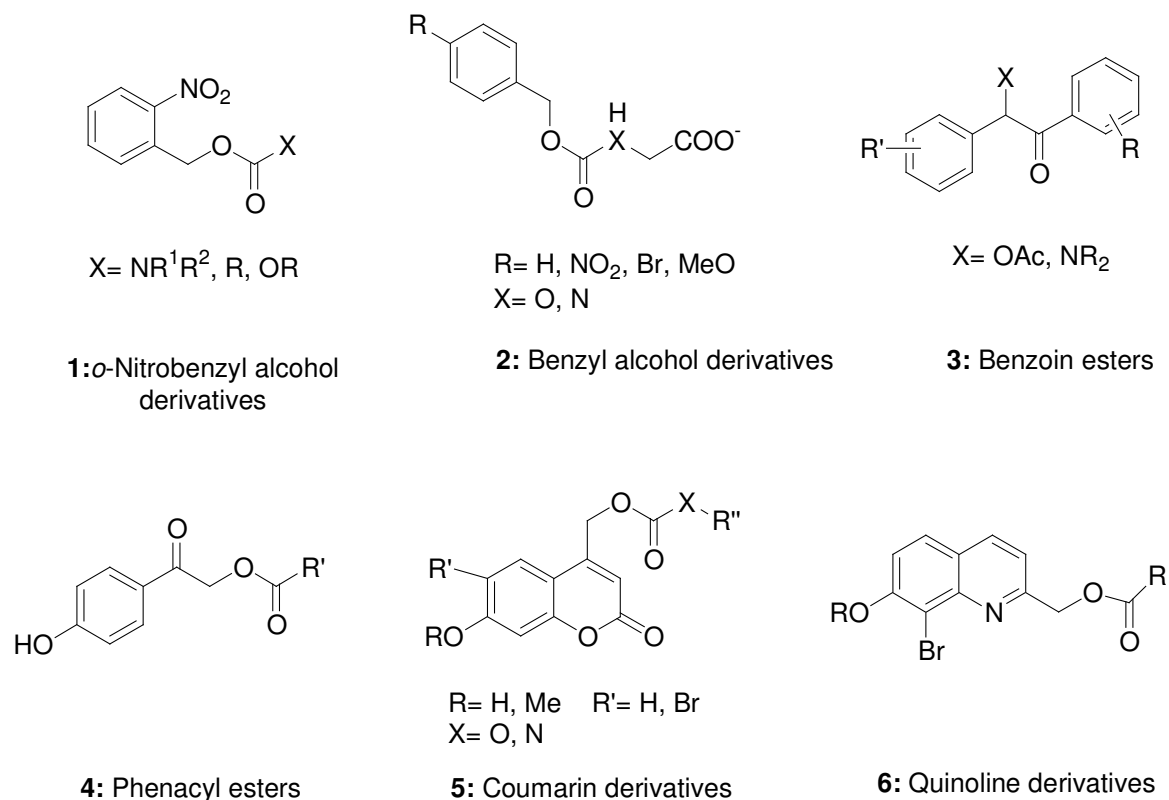
The first caged compound, P^3-1-(2-nitro)phenylethyl-adenosine triphosphate (caged ATP) was synthesized and single-photon photolyzed by Kaplan, et al.[1] With the shuttered Hg arc light source that they used, they were able to study only slower processes, on 1-s and 100-ms time scales. A laser was first used for this caged compound in the late 1970s by McCray.[42] A passive dye Q-switched, frequency-doubled ruby laser was used for this research, which demonstrated that biochemical kinetics inside cells could be followed on the ms-time scale.

Although they could focus a laser beam through a microscope objective to a very small area in two-dimensions, on the order of a few $(\mu m)^2$, for one-photon photolysis of caged compounds in or near biological tissues, a serious problem arises because photodamage of the biological material occurs. This damage is due to considerable absorbance by proteins and nucleic acids at short wavelengths, so multiphoton excitation provides a good method to reduce the damage.

Infrared (IR) light and multiphoton excitation have been used as a less damaging method for the photolysis of caged compounds (Figure 4). Multiphoton excitation (usually two-photon excitation) provides an excellent way to photolyze the caged compounds with high spatial resolution in living cells. Because the non-linear (quadratic) two-photon absorption is confined to the focus of the laser beam, the light-induced uncaging processes are localized in this small volume. For a two-photon photolysis experiment, the successful wavelength used in one-photon photolysis experiment is approximately doubled. At very high intensities, a normal UV-chromophore can be excited by two IR photons. Also, because cells and tissues are relatively transparent to IR light, there is much less photodamage, light absorption and scattering, with much deeper penetration in this photolysis processes. Molecular two-photon excitation (2PE) was first predicted by Maria Göppert-Mayer in 1931.[43] Two-photon excitation requires high peak intensities, typically 10^{20} to 10^{30} photons/(cm^2· s) for the observation of two-photon absorption. Two-photon excitation for biological imaging and two-photon uncaging of biomolecules was first demonstrated in 1990 by Denk, Strickler and Webb.[44] They suggested that it would be better to use lower energy IR photons than UV photons. In one-photon photolysis, UV photons that are not involved in uncaging can photo-oxidize proteins, leading to damage, while the excess IR photons that are not involved in uncaging can only be absorbed into vibrational states of proteins, leading to at most a temperature increase of the sample. The highest two-photon absorption

probability is in the region of highest peak power density and the peak power is low outside this region. The two-photon photolysis is highly localized into a small volume and the photodamage is minimized. The most commonly used laser is the argon ion-pumped, titanium-sapphire solid-state pulsed laser that gives femtosecond laser outputs at wavelengths above 700 nm. It is easier for non-laser specialists to use this type of laser to apply two-photon photolysis to the compounds that have UV-absorption above 350 nm.

Figure 4. The Three-dimensional Spatial Selectivity of Single vs. Two-photon Excitation
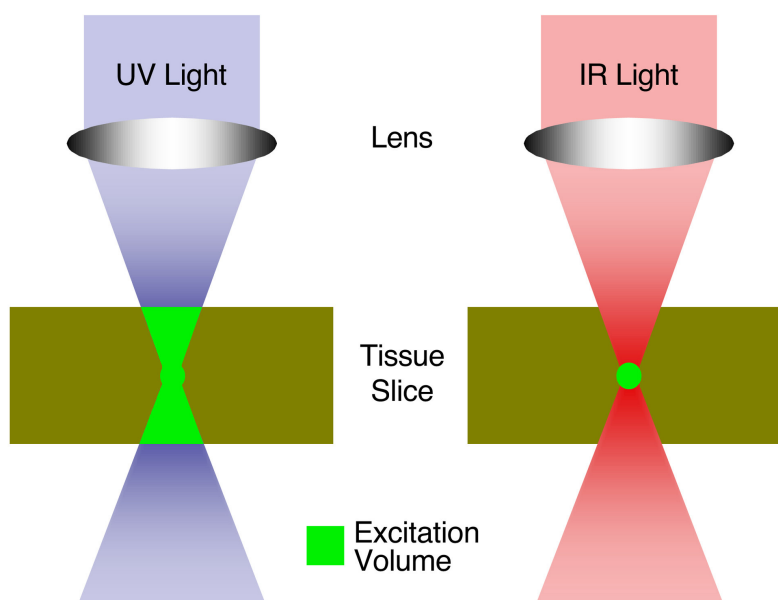
The two-photon uncaging action cross-section, δ_u, is a measure of the sensitivity of a chromophore to two-photon photolysis. It is the product of the two-photon absorbance cross-section, δ_a, and the uncaging quantum yield, Q_{u2}. To be useful in biological systems, δ_u should exceed 0.1 GM (Goeppert-Mayer, 10^{-50} cm^4 s/ photon).[35] To measure the two-photon uncaging cross-sections, δ_u, Furuta and Tsien[35] illustrated a method of using fluorescein as a reference

compound whose two-photon fluorescence quantum yield (Q_{f2} = 0.9 mol/ein) and absorbance cross-section (δ_{aF} = 30 GM at 740 nm)[45] are known. A value of δ_u is calculated from the following equation:

$$\delta_u = \frac{N_P \phi Q_{f2} \delta_{aF} C_F}{\langle F(t) \rangle C_s}$$

Where N_p is the number of product molecules formed per unit time (molecules/s, determined by HPLC analysis); ϕ is the collection efficiency of the detector used to measure the fluorescence of fluorescein emitted at a right angle to the beam and passed though a 535/45 nm bandpass filter; C_F is the concentration of fluorescein (mol/L); $\langle F(t)\rangle$ is the time averaged fluorescent photon flux (photons/s) collected by the detector; and C_s is the initial concentration of caged substrate (mol/L).[35]

The design and synthesis of two-photon sensitive caged biomolecules that can be used inside living cells is challenging for organic chemists. Only the photoremovable protecting groups that possess sufficiently large two-photon uncaging action cross-sections, δ_u, have potential for biological applications.[35] As an added advantage, chromophores with sensitivity to multiphoton excitation tend to be highly sensitive to single-photon excitation. That means if the caged compound has a large two-photon uncaging action cross-section, it also has high one-photon uncaging quantum efficiency. Although *o*-nitrobenzyl-type groups have been most widely used as photoremovable protecting groups, recently, coumarin derivatives were reported as more two-photon sensitive caged compounds.[35,36,46-49] For example, 6-bromo-7-hydroxycoumarin-4-ylmethyl has been used as a multiphoton-sensitive protecting group for

neurotransmitters, DNA and RNA, diols, and an inhibitor of nitric oxide synthase. MNI-glutamate has been reported to release glutamate upon two-photon excitation in sufficient quantities to be useful for investigating the function of glutamate receptors. The calcium cage azid-1 can effectively release calcium under two-photon excitation.[50] A potentially biologically useful multiphoton-sensitive protection group is 8-bromo-7-hydroxyquinoline.[51]

Caged compounds have been used to study the fast kinetics of signal transduction, such as with neurotransmitters, but they have not been widely used to regulate slower processes such as gene expression or protein synthesis. Furthermore, the use of multiphoton excitation to mediate the release of biological effectors has not found much widespread application, despite its advantages over single photon processes. Further expansion of the prospects of applications of caged compounds will enable less invasive mapping of local responses to the messengers involved in signal transduction, gene expression, and protein synthesis. This technology has the potential to become a powerful drug delivery method that can be used as a research tool and for therapy.

PART 2. LIGHT-SWITCHABLE GENE EXPRESSION

Understanding physiological processes is a major requirement for modern scientists to develop gene therapy. How to regulate the expression of transferred exogenous genes within the human body is a serious problem. By specially mutating the genetic code, we can observe the microscopic details of cellular functions and learn how the genes work. Several regulatable transgene systems that have been created for eukaryotes, and this technique has yielded a tremendous amount of information about physiological function. Among these, using small molecules to control gene expression in complex biological systems is a powerful tool to study gene function. They can control gene expression in time and they are more practical for real gene therapy to regulate gene expression than using heat shock or heavy metals.[52,53]

In order to improve temporal and spatial resolution and provide better control of absolute expression levels, light-switchable gene expression systems have been reported. For example, phytochrome controls gene expression by reversibly interconverting between its inactive and active forms.[54] A light-switch gene promoter system developed by Sae Shimizu-Sato[55] can be rapidly and reversibly induced by a flash of light. Their achievement is based on the two forms of holoprotein reversibly interconverted by exposure to IR light.[56] It is hard to find general application of this technique. Ando[57] reported the photolysis of caged RNA/DNA to regulate gene expression in zebrafish embryos. The first example of using a caged small molecule to control gene expression was reported by John Koh, et al[58] in 2000. This regulation was achieved by using hormone analogues whose agonist properties are blocked by a photoremovable protecting group. Another light-activatable ecdysone-inducible gene expression system has been created in Lawrence's lab.[59]

Because multiphoton photolysis can provide high localization, less damage to tissue, minimized scattering, and deeper penetration to regulation of gene expression, multiphoton excitation of caged compounds could be an invaluable tool for these purposes. We can use multiphoton sensitive photoremovable protecting groups to cage biological active molecules and release them by photolysis. Our plan is to create a light activated gene expression system for zebrafish based on the use of caged regulators of these processes. Photochemical control of gene expression in a complex biological system, such as zebrafish, would be a significant method for investigating gene functions involved in a multitude of physiological processes: signal transduction, cell-to-cell communication, neuronal signaling, cell cycle regulation, development, motility, and many others. A system that could be mediated by a multiphoton excitation process (high localization, less damage to tissue, minimized scattering, deeper penetration) would have the additional advantage of facilitating control at the single cell level deep inside tissue, which will be especially useful for studying vertebrate development. Zebrafish has been widely used to study vertebrate development because it has two major advantages: (1) Zebrafish have transparent bodies, which make it possible to monitor their tissues and neurons in vivo; (2) Zebrafish are small and they are easy to maintain and reproduce them in the research laboratory.

In order to prevent constitutive gene expression, a distinct regulator gene can be cotransferred to govern the expression of the target gene. In 1994, Wang *et al.*[60] developed a regulatory system, which can be used in both animal and human gene transfer studies. This system can be switched on/off in response to a small chemical compound, such as mifepristone (RU486). Because the physiological properties of mifepristone have been well studied, creating a caged mifepristone to regulate a light-switchable gene expression system with multiphoton excitation is practical. Mifepristone can regulate the gene expression system by an

autoregulatory feedback loop. At lower concentrations, mifepristone acts as an agonist on the progesterone receptor.

Wang's system has been commercialized by Invitrogen under GeneSwitch[TM]. A photochemically activated system, works as shown in Figure 5. The GeneSwitch[TM] system includes pGene/V5-His, pSwitch, mifepristone, and a control expression plasmid containing the lacZ gene and pGene/V5-His/lacZ. In the absence of mifepristone, the translation of the GAL4-DBD/hPR-LBD/p65-AD fusion gene by the pSwitch regulatory vector is controlled by the minimal thymidine kinase (TK) promoter. This kind of expression only provides inactive protein (GeneSwitch[TM]) in the nucleus. In this system, mifepristone binds to the receptor at the nanomolar level ($K_d \sim 3$ nM), and causes a conformation change in the hPR-LBD. Mifepristone can activate expression of the gene of interest by dimerizing and binding GeneSwitch[TM] protein (GAL4-DBD/hPR-LBD/p65-AD) to GALUAS. The ligand-bound protein can regulate the expression of its own gene to synthesize GeneSwitch[TM] protein by activation of an autoregulatory feedback loop, therefore only a small amount of uncaged compound (~ 0.1 nM) is necessary to activate gene expression.[60] Modified with a 42-amino acid deletion in the progesterone receptor-ligand binding domain (hPR-LBD), the system is not activated by endogenous progesterone. We expect the photolabile-protecting group Bhc-diol can deactivate mifepristone, and Bhc-diol-mifepristone can diffuse into cells. When the cells are exposed to a flash of light, the physiological activity of mifepristone can be reactivated.

The control of gene expression in complex biological systems would be invaluable for studying physiological processes. Because of the advantages afforded by multiphoton excitation, further development of applications for the use of photoremovable protecting groups capable of releasing biomolecules through multi-photon processes will fuel efforts to answer questions

about the temporal and spatial relevance of signaling by physiological messengers. It will impact many areas of biology and medicine, including, but no limited to, developmental biology, neuroscience, pharmacology, molecular biology, and medical diagnostics. Moreover, the development of a light activated gene expression system should be particularly useful for studying development and gene function in zebrafish.

Figure 5. Photoactivated Gene Expression Based on GeneSwitch[TM]
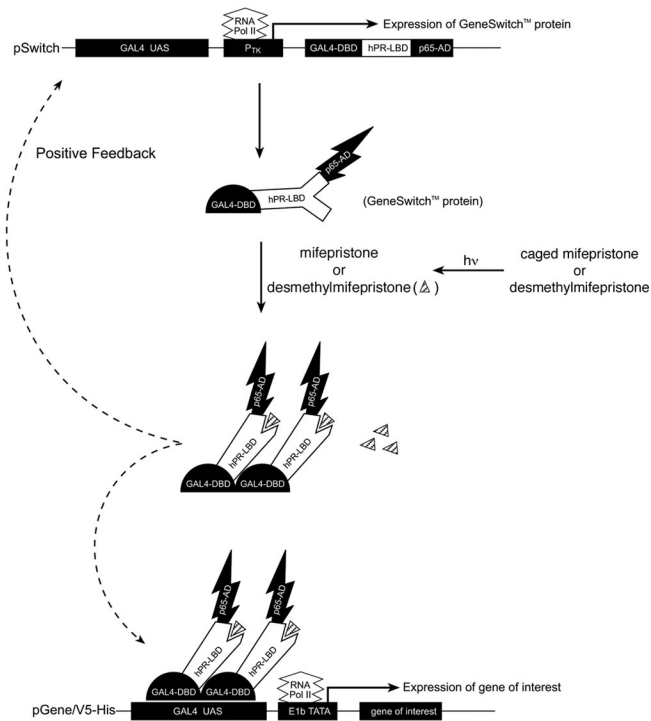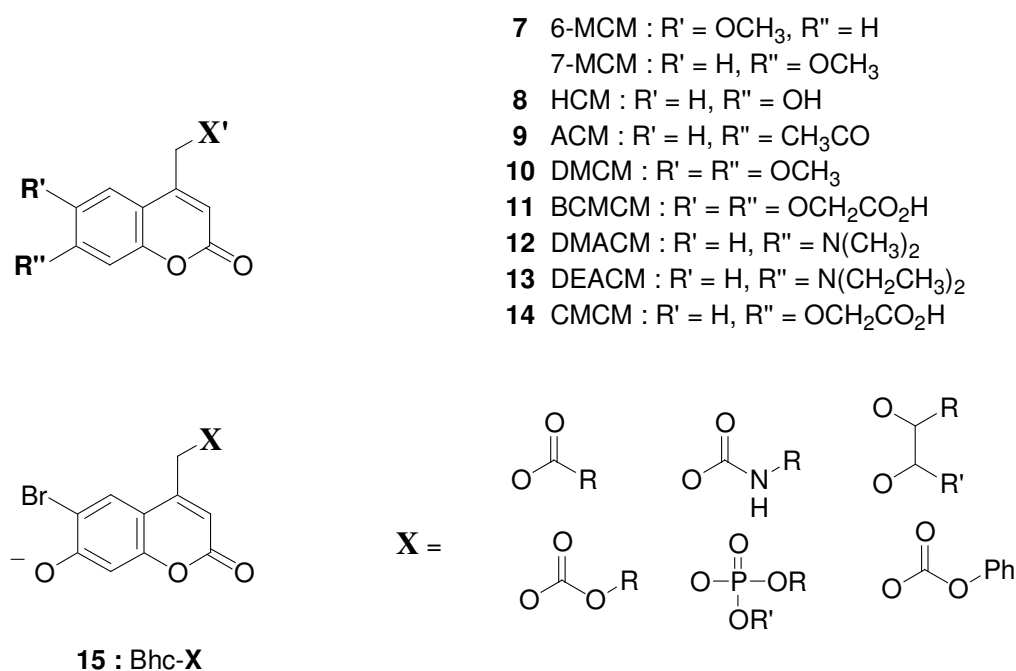
CHAPTER 2

RESULTS AND DISCUSSIONS

PART 1. DEVELOPMENT OF A NEW PHOTOREMOVABLE PROTECTING GROUP FOR

ALDEHYDES AND KETONES

The coumarin-based caged compounds have received wide attention due to their ability to mediate biological activities in animal cells with light. Although the 4-coumarinylmethyl group has been used as a fluorescent tag for biological molecules,[33] this highly fluorescent group had not been used as a caging group until Toshiaki Furuta et al. illustrated a method for the synthesis of esters of diethyl phosphate and cAMP.[61] 4-(7-methoxycoumarinyl)methyl (**7**, MCM),[40,49] 4-(7-hydroxycoumarinyl)methyl (**8**, HCM),[34,62] 4-(7-acetoxycoumarinyl)methyl (**9**, ACM),[34,62] 4-(6,7-dimethoxycoumarinyl)methyl (**10**, DMCM),[46] 4-(6,7-biscarboxymethoxycoumarinyl)methyl (**11**, BCMCM),[36,63] 4-(7-dimethylaminocoumarinyl)methyl (**12**, DMACM),[36,63-65] 4-(7-diethylaminocoumarinyl)methyl (**13**, DEACM),[36,63-65] 4-(7-carboxymethoxycoumarinyl)methyl (**14**, CMCM),[66] and 4-(6-bromo-7-hydroxycoumarinyl)methyl (**15**, Bhc)[35,57,67-71] have been successfully used to "cage" carboxylates, amines, diols, phosphates, and alcohols and phenols.[71] Furthermore, Bhc (**15**) caged compounds can be photolyzed under two-photon excitation with a large absorbance cross-section[35,57,67-70] (Figure 6).
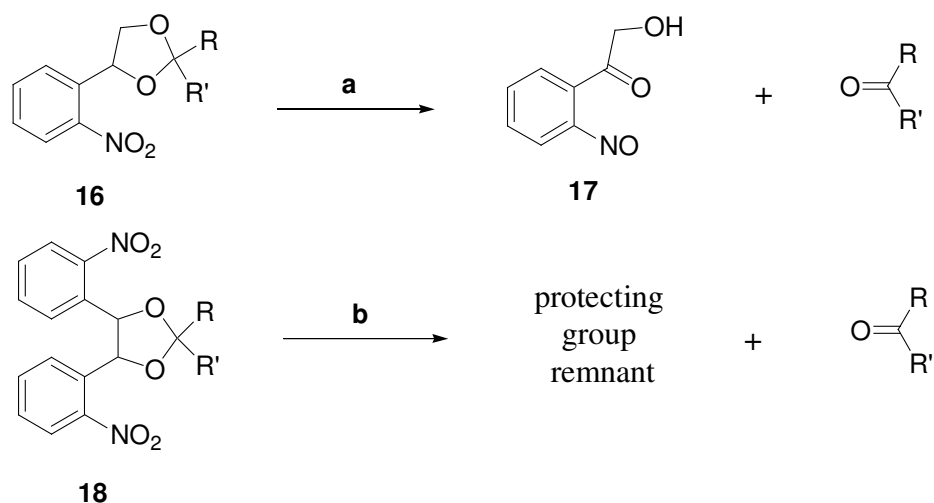
Although many photoremovable protecting groups are known, such groups for ketones and aldehydes are limited, especially under physiological conditions.[72-76] This is surprising because carbonyl groups are one of the most common functional groups in organic compounds

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and biological effectors, especially drugs. Synthetically useful photoremovable protecting groups for carbonyl groups such as *N,N*-dimethylhydrazones[77] require the generation of singlet oxygen, while others require a triplet sensitizer, as in the case of dithioacetals.[72,78] Both methods cannot be used in biological systems. *o*-Nitrophenylethylene glycol derivatives (**16**)[79] and (**18**)[73,80] release carbonyl compounds upon exposure to 350 nm light in organic solvents (Scheme 1). Similarly, polymer-supported *o*-nitrophenylethylene glycols[74] offer photoremovable protection to aldehydes, releasing them both in benzene and in a stream of air after exposure to a visible-light mercury lamp for 7 h. The photolysis is not efficient, so it is impractical to apply this photoremovable protecting group to biological tissues. These protecting groups require significant synthetic adaptation for physiological use, and they would still suffer from very poor sensitivity to multiphoton excitation.

Figure 6. Coumarin Based Photoremovable Protecting Groups and Bhc with Biological Utility.

7 6-MCM : R' = OCH$_3$, R" = H
7-MCM : R' = H, R" = OCH$_3$
8 HCM : R' = H, R" = OH
9 ACM : R' = H, R" = CH$_3$CO
10 DMCM : R' = R" = OCH$_3$
11 BCMCM : R' = R" = OCH$_2$CO$_2$H
12 DMACM : R' = H, R" = N(CH$_3$)$_2$
13 DEACM : R' = H, R" = N(CH$_2$CH$_3$)$_2$
14 CMCM : R' = H, R" = OCH$_2$CO$_2$H

15 : Bhc-**X**

X =

Scheme 1. *o*-Nitrophenylethylene Glycol as Photoremovable Protecting Groups for Aldehydes

and Ketones

(a) hν (350 nm), benzene, 6 h, 31-90%; (b) hν (350 nm), CH₃CN or Et₂O, 1-2 h, 69-97%

Using Bhc as a basis, we set out to prepare a good multiphoton-sensitive photoremovable protecting group that can release biologically active ketones or aldehydes in living cells, tissues, and animals. The general idea that acetals and ketals of 6-bromo-4-(1,2-dihydroxyethyl)-7-hydroxycoumarin (**19**, Bhc-diol-acetals/ketals) would be capable of releasing aldehydes and ketones upon single- or two- photon photolysis under simulated physiological conditions such as KMOPS buffer (100 mM KCl and 10 mM MOPS titrated to pH 7.2 with KOH) is outlined in Scheme 2. By studying the (7-methoxycoumarin-4-yl)methyl-caged phosphates, carboxylates, and sulfonates, Bendig et al. proposed a photochemical S_N1 reaction (solvent-assisted photoheterolysis) mechanism for the coumarin-type photoremovable protecting groups.[40] Upon photolysis of Bhc under physiological conditions, zwitterion **22**, a possible intermediate suggested by Bendig et al., is generated from the diradical **21** by rapid intramolecular single

electron transfer. Bulk solvent (H_2O or ^-OH) trapping of the resulting cation followed by dissociation to Bhc-diol (**20**) and the carbonyl compound is competitive with recombination to regenerate the stating ketal.

Scheme 2. Photolysis of Bhc-diol-Protected Aldehydes and Ketones
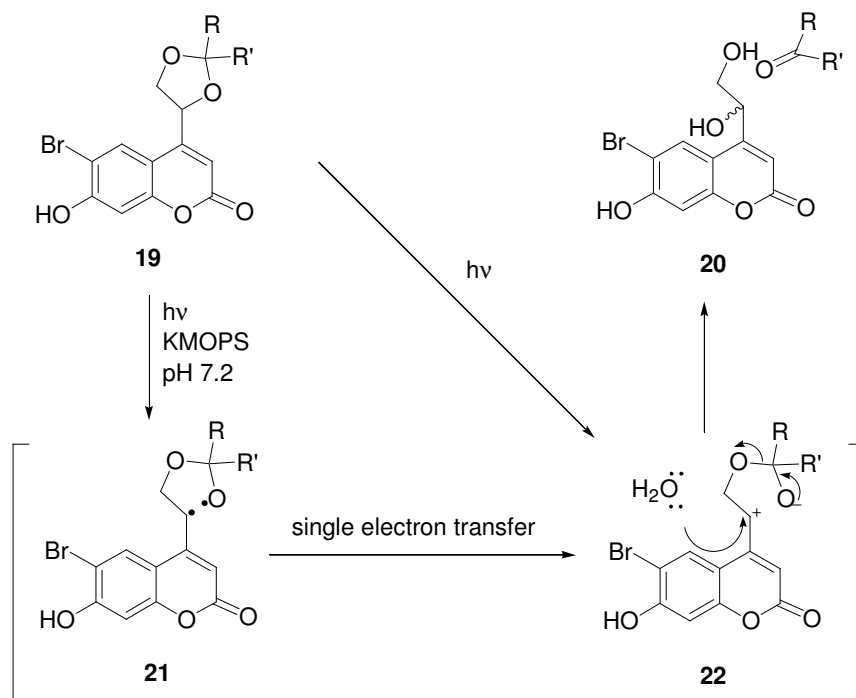
We have devised a method to accomplish this using Bhc-diol (**20**), which we have synthesized in five steps from commercially available materials (Scheme 3).

4-(6-Bromo-7-hydroxycoumarinyl)methyl chloride (**25**, Bhc-Cl) was prepared in 90% yield by a Pechman condensation of 4-bromoresorcinol (**23**) with ethyl-4-chloroacetoacetate (**24**) in concentrated sulfuric acid[35]. The phenolic hydroxy group of Bhc-Cl was protected as the acetate by using acetic anhydride and pyridine, followed by formation of the triphenyl phosphonium salt **27** in acetonitrile at 85 °C with triphenylphosphine overnight. Olefination[81] of

the 6-bromo-7-hydroxycoumarin triphenyl phosphonium salt was accomplished by a Wittig reaction with aqueous formaldehyde, using sodium carbonate as the base. The reaction proceeded with loss of the acetate protection group at the same time to reveal Bhc-vinyl (**28**). Osmium teteroxide dihydroxylation of the 6-bromo-7-hydroxy-4-vinylcoumarin (**28**) provided Bhc-diol (**20**).

Scheme 3. Synthesis of Bhc-diol
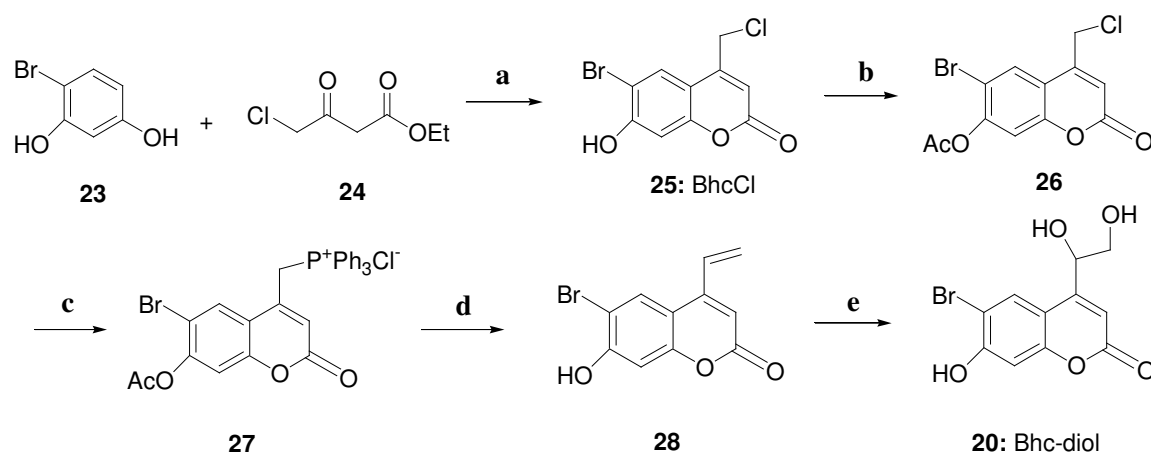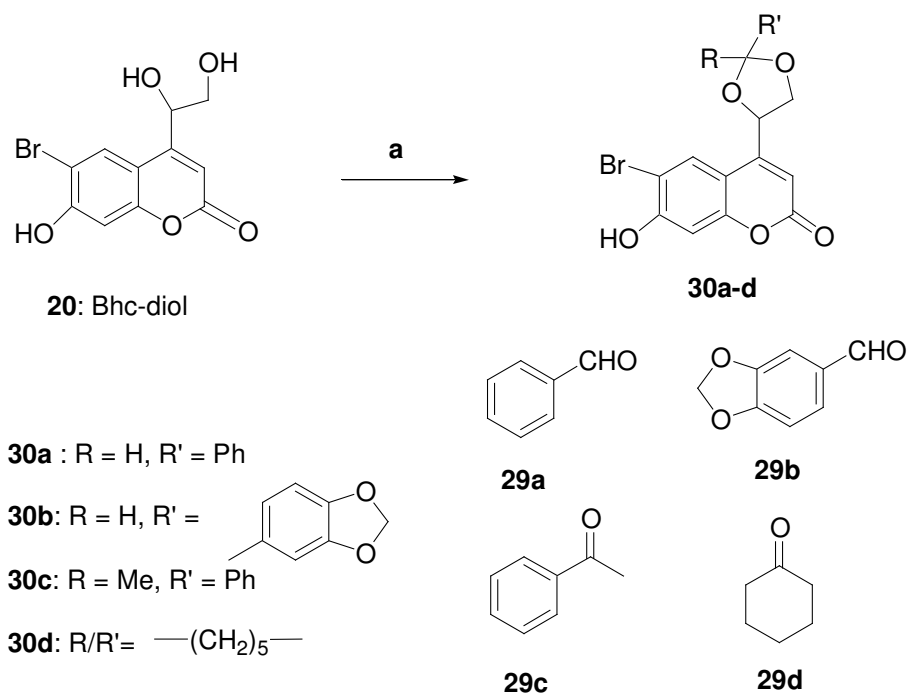

Reagents and Conditions:
(a) H_2SO_4, 6d, 90%; (b)pyridine, acetic anhydride,85%; (c) Ph_3P, CH_3CN, 82%;
(d) CH_2O, H_2O, Na_2CO_3, 72%; (e) H_2O, OsO_4, acetone, N-methylmorpholine N-oxide, 80%

Acetalization with benzaldehyde and piperonal and ketalization with acetophone and cyclohexanone provided four caged compounds with which to test the effectiveness of photochemical release of ketones from Bhc-diol. The Bhc-diol-protected aldehydes and ketones were synthesized as shown in Scheme 4.

Bhc-diol was refluxed in toluene with benzaldehyde (**29a**), piperonal (**29b**), acetophenone (**29c**), or cyclohexanone (**29d**) in the presence of pyridinium p-toluene-sulfonate (PPTS); solid magnesium sulfate was added to remove water. This afforded Bhc-diol-acetals and Bhc-diol-ketals as a mixture of diastereomers (in the case of **30a-c**) in modest yields.

Scheme 4. Synthesis of Bhc-diol-protected Aldehydes and Ketones

20: Bhc-diol

30a-d

30a : R = H, R' = Ph

30b: R = H, R' =

30c: R = Me, R' = Ph

30d: R/R'= —(CH₂)₅—

29a

29b

29c

29d

(a) **29a-d**, pyridinium *p*-toluenesulfonate, MgSO₄ (anhydrous), toluene, BuOH, reflux, 110°C, 8h, 57%, 28%, 22%, 39% for **30a-d**, respectively.

Each of the Bhc-diol-protected compounds was tested in vitro for their optical properties (UV spectrum, extinction coefficient). Stability and its resistance to spontaneous hydrolysis in the dark under simulated physiological condition can be measured readily by HPLC, looking for disappearance of starting Bhc-diol-protected aldehydes and ketones and appearance of the caging group remnants. A small amount (2-5%) of decomposition of the conjugate was observed after seven days in pH 7.2 KMOPS buffer (Figure 7).

Figure 7. Time Courses of Spontaneous Hydrolysis of the Bhc-diol-protected Aldehydes and

Ketones in the Dark under Simulated Physiological Condition
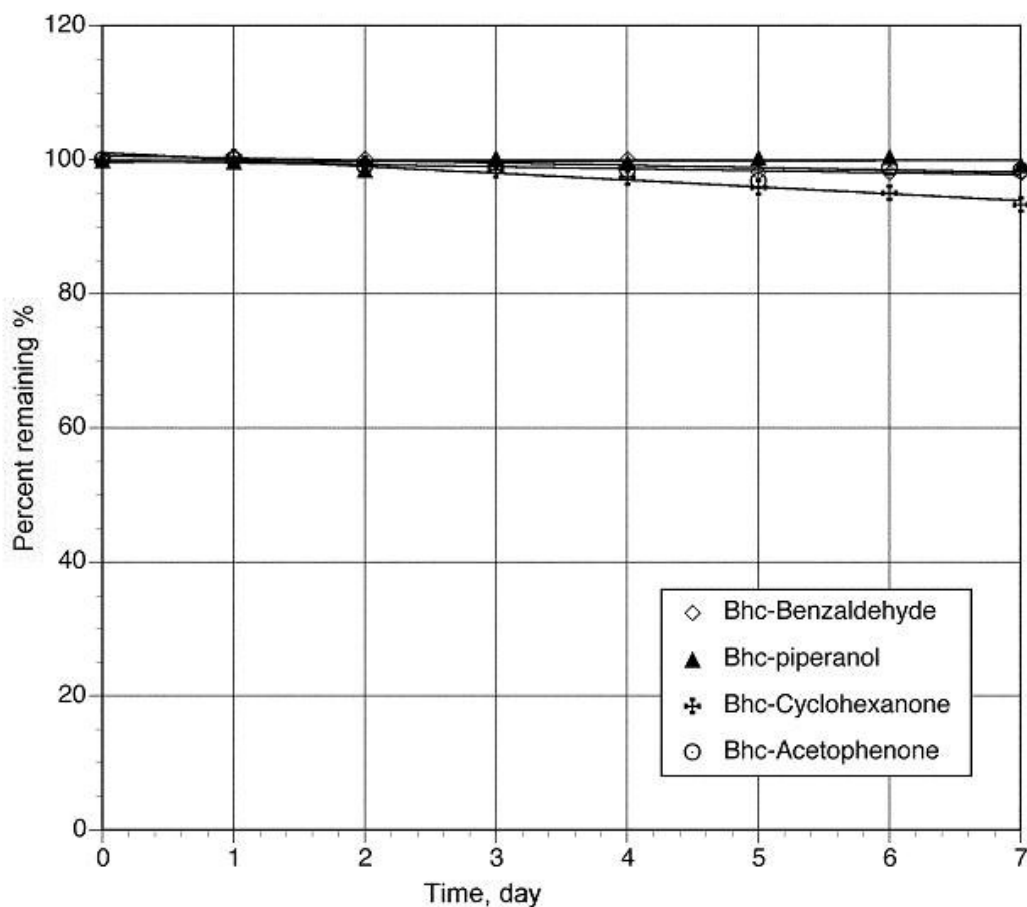
Irradiation of Bhc-diol-protected aldehydes and ketones with 365 nm light in pH 7.2 KMOPS buffer released the free aldehydes and ketones (Figure 8). The photochemical experiments were carried out by using a UV lamp and taking aliquots for HPLC analysis at periodic intervals. Comparing the time courses for these reactions reveals that Bhc-diol-benzaldehyde and Bhc-diol-piperonal were photolyzed slightly more efficiently than the acetophenone and cyclohexanone derivatives. All of these four Bhc-diol-protected acetals/ketals can be fully deprotected by photolysis in two minutes. From these data, single-photon uncaging

quantum efficiencies, Q_{u1}, were determined as previously described, $Q_{u1} = (I \cdot \sigma t_{90\%})^{-1}$.[82,83] The UV intensity of the lamp I was measured by using potassium ferrioxalate actinometry[83] in the same setup. Release of piperonal or acetophenone was monitored at 313 and 240 nm, respectively, and the progress curves plotted as an exponential rise to max. Concentrations were determined using an external standard.

Figure 8. The Time Courses of One-photon Photolysis of Bhc-diol-protected Aldehydes and Ketones

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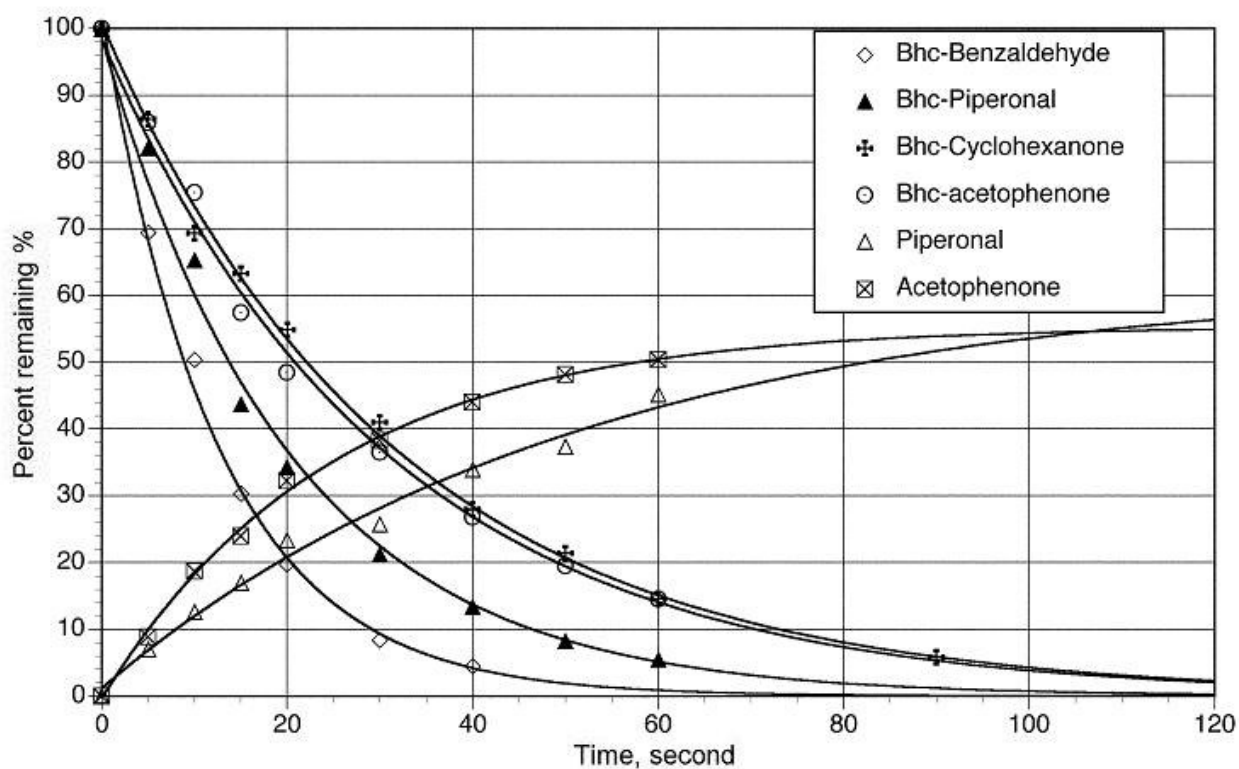
Calculation of One-Photon Quantum Efficiency

$Q_{u1} = (I \cdot \sigma \cdot t_{90\%})^{-1}$

I - lamp intensity (ein×cm^2/s) (determined by potassium ferrioxalate actinometry)
ε - molar extinction coefficient (cm^{-1} M^{-1})
σ - decadic extinction coefficient (10^3 times ε, cm^2/mol)
$t_{90\%}$ - irradiation time in second for 90% conversion to product

---

One-photon quantum efficiencies of Bhc-diol-protected aldehydes and ketones are summarized in Table 1 along with selected absorption data. The quantum efficiencies for single-photon photolysis of Bhc-diol-protected benzaldehyde, piperonal, acetophenone, and cyclohexanone are similar to those of other Bhc-protected compounds.[35,51,57,68,69]

Table 1. Photochemical Properties of Bhc-ketals or acetals

Bhc-ketals or acetals	λ_{max} (nm)	ε_{365} (M^{-1} cm^{-1})	ε_{370} (M^{-1} cm^{-1})	Q_{u1} (mol/ein)	$\delta_u (GM)^*$ (740nm)
Bhc-diol-benzaldehyde	370	18000	18500	0.057	0.90
Bhc-diol-piperonal	370	19500	19800	0.035	0.60
Bhc-diol-cyclohexanone	370	12600	12800	0.032	0.51
Bhc-diol-acetophenone	370	18000	18400	0.030	1.23

*GM=10^{-50}cm^{4}s mol^{-1}
To be useful in biological systems, $\delta_u > 0.1$ GM

The extent of carbonyl compound release as measured by HPLC was half of what was expected. To explore the possibility of secondary photochemical degradation, KMOPS-buffered solutions of piperonal and acetophenone were each exposed to 365 nm light for one min, but neither compound showed any signifant decomposition. Irradiation of a 1:1 mixture of Bhc-diol

and piperonal or acetophenone in KMOPS buffer also showed no change in concentration of the carbonyl compounds. Degradation of the carbonyl compounds after uncaging appears to not be the result of a direct or a Bhc-diol-mediated photochemical process.

The two-photon uncaging cross-section, δ_u, of Bhc-diol-protected aldehydes and ketones was measured as an external standard as previously described.[51] The experimental setup is shown in Figure 9. Measurements were carried out in microcuvettes (10 × 1 × 1 mm illuminated dimensions) with an effective filling volume of 20 μL using light from a fs-pulsed, mode-locked Ti:Sapphire laser focused on the center of the cuvette chamber with a 25 mm focal length lens optimized for IR lasers. The two-photon uncaging cross-section (δ_u) was estimated by referencing to fluorescein, a compound with a known two-photon fluorescence quantum yield (Q_{f2} = 0.9 mol/ein) and absorbance cross-section (δ_{aF} = 30 GM at 740 nm).[47] The progress of the uncaging reaction was measured by HPLC and graphed as a function of time (Figure 10).

Figure 9: Apparatus for the Measurement of Two-photon Uncaging Cross-sections

<div style="border:1px solid black">
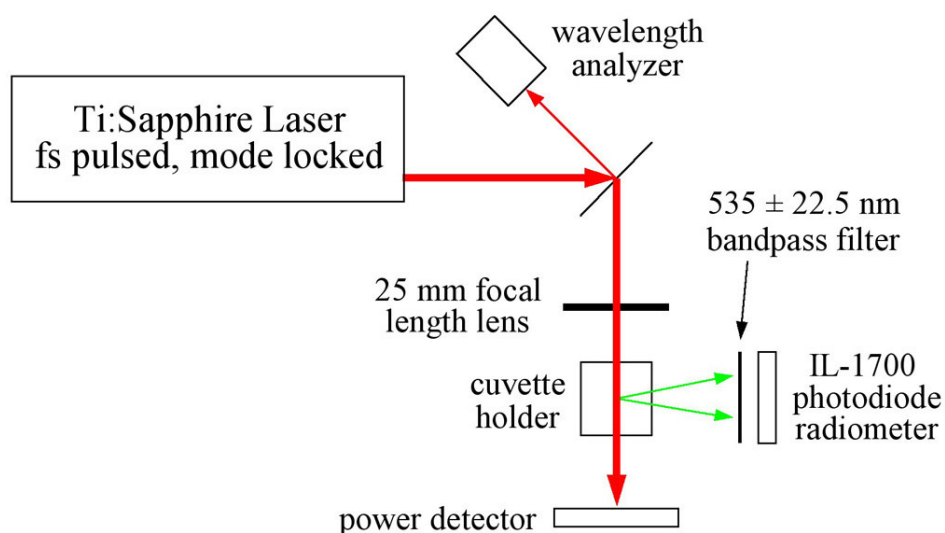
Calculation of Two-Photon Uncaging Action Cross-Section δ_u

$$\delta_u = \frac{N_p \phi Q_{f\,2} \delta_{aF} C_F}{\langle F(t)\rangle C_s}$$

N_p-number of product molecules formed per unit time
C_F-the concentration of fluorescein
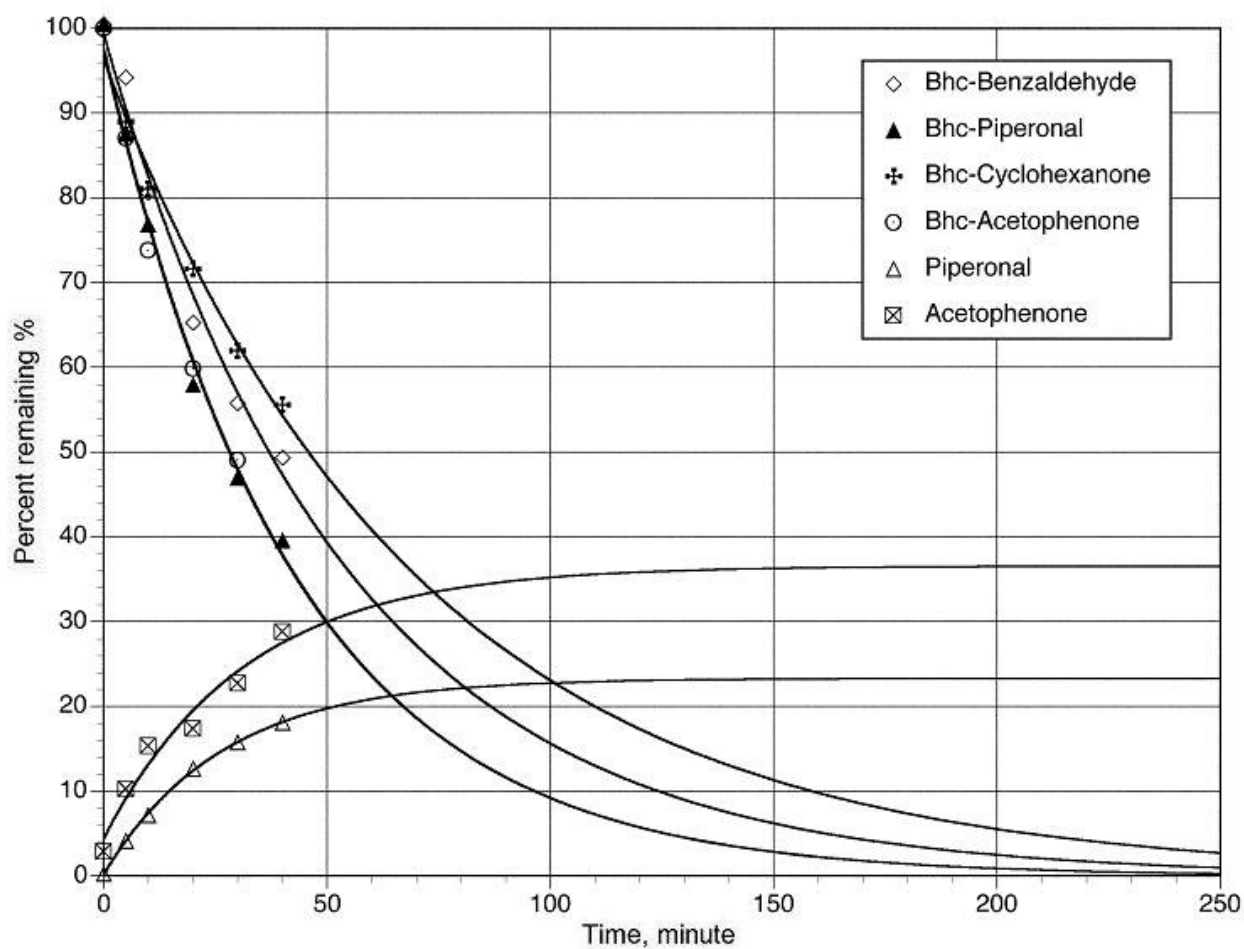Φ-the collection efficiency of the detector
δ_{aF}-the fluorescein absorbance cross-section
Q_{f2}-the two-photon fluorescence quantum yield of fluorescein
$<F(t)>$-the time averaged fluorescent photon flux
C_s-the initial concentration of caged substrate

</div>

Figure 10. The Time Courses of Two-photon Photolysis of Bhc-diol-protected Aldehydes and

Ketones

The values of δ_u (Table 1) determined for Bhc-diol-acetals/ketals are similar to the values obtained for other Bhc-protected compounds. Any discrepancies are probably a result of differences in laser power and the optical setups employed. The longer time constants (min), as compared to the single-photon kinetics (s), are due to the small volume of the sample that is actually irradiated (~1 fL) relative to the bulk solution (20 μL). Sufficient quantities of the Bhc-diol-acetals/ketals for HPLC analysis must diffuse into the laser's focal volume, where uncaging efficiency is high, undergo photolysis, and then diffuse back out into the bulk solution.

The results indicate that Bhc-diol can be used as a photolabile protecting group to cage biologically active messengers containing carbonyl functionality. Since Bhc has been used in biological systems, this represents the first example of a photolabile protecting group capable of releasing aldehydes and ketones by single- and two-photon excitation under physiological conditions.

PART 2. LIGHT-SWITCHABLE GENE EXPRESSION SYSTEM

After we achieved the above accomplishment, we began to synthesize the desired Bhc-diol-protected biological messengers, such as mifepristone, that have a carbonyl group in their structures (Figure 11).

Important gene messengers such as the progesterone receptor antagonist, (**31, mifepristone**, also known as RU486) (synthetic steroid), induce gene expression in mammalian cells.[60] Because mifepristone's carbonyl group is important for its action on the progesterone receptor, Bhc-diol can be used as a photoremovable-protecting group for releasing mifepristone inside living cells, tissues, and animals. As the physiological properties of mifepristone are well known, it is ideal to induce mammalian cells with Bhc-diol caged mifepristone.

Figure 11. Mifepristone and Bhc-diol-mifepristone

31: Mifepristone (RU486)
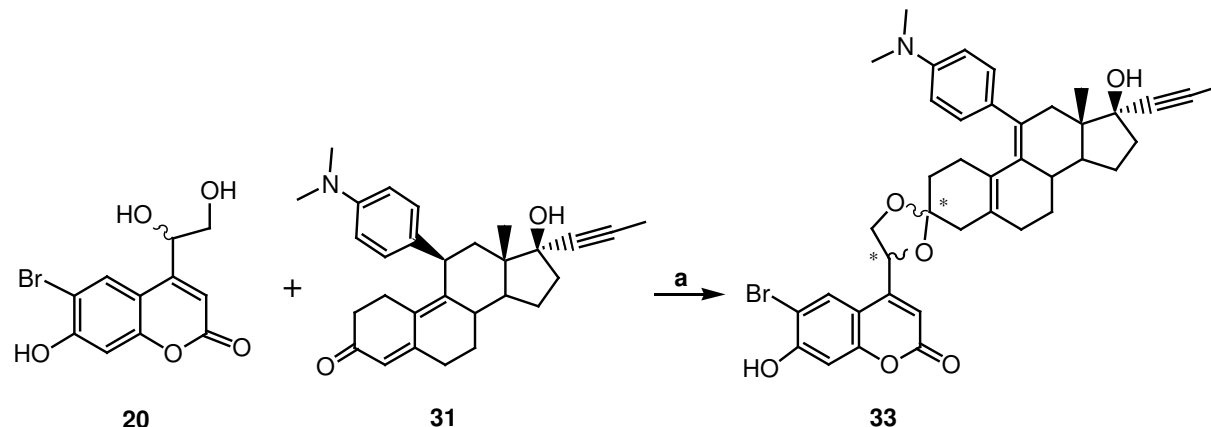
32: Bhc-diol-mifepristone
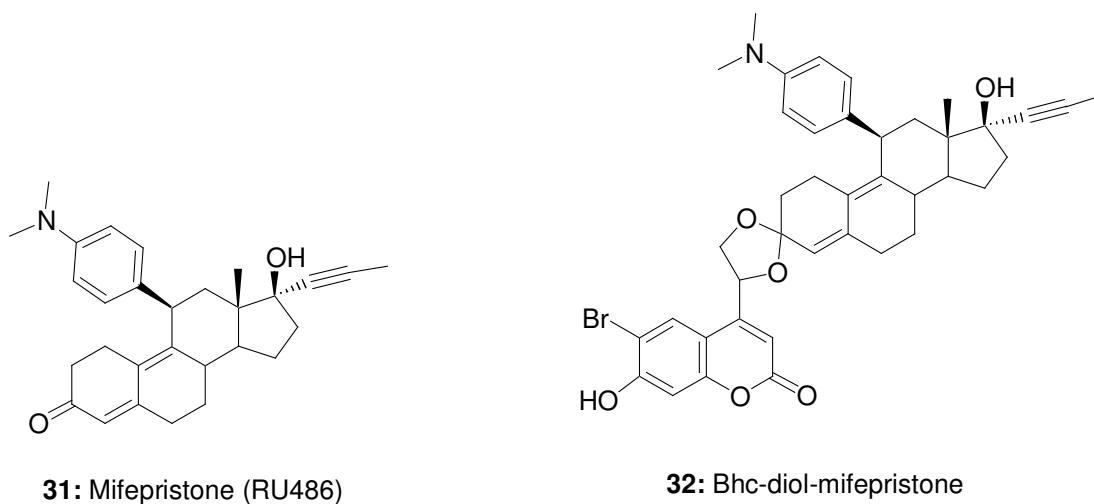
The previous ketal functionalization is affected with Bhc-diol in an acid medium. Because mifepristone is a relatively complicated molecule with several functional groups, we have expected that there would be some problems to prepare the Bhc-diol caged mifepristone (**32**).

The standard method of making ketals from ketones and 1,2-diols is by refluxing the substrates with pyridinium *p*-toluenesulfonate in dry toluene and anhydrous magnesium sulfate to remove the water generated. A trace quantity of Bhc-diol-mifepristone derivative has been synthesized in our lab, having carried out the reaction with Bhc-diol and mifepristone. The mass spectrum and NMR data indicate that we have produced the caged mifepristone as a mixture of diastereomers in very low yield with migration of conjugated doubled bonds.

After making numerous efforts to modify the reaction condition, such as changing the solvent from toluene to benzene, using *p*-toluenesulfonic acid instead of pyridinium *p*-toluenesulfonate, and using molecular sieves instead of $MgSO_4$, there was no improvement.

Other more efficient methods to make ketals of α, β-unsaturated ketones were examined. Several methods were tried, such as microwave ketalization and TMSOTf catalysis, and TMSCl catalyzed ketalzation.[84] Under TMSCl condition, THF was used as the solvent to make Bhc-diol dissolve in the reaction mixture. This reaction gave Bhc-diol-mifepristone derivative **33** in good yield (Scheme 5).

HPLC analysis indicated that the product of the ketalization of Bhc-diol and mifepristone with TMSCl is a mixture of four similar compounds that have different retention time on HPLC. From LC-Mass data, they have the same molecular weight, 712/714, which is the desired molecular weight of the product. UV spectral analysis also gave very similar data that has two major absorptions, at 288 nm and 326 nm.

Scheme 5. Synthesis of Bhc-diol-mifepristone

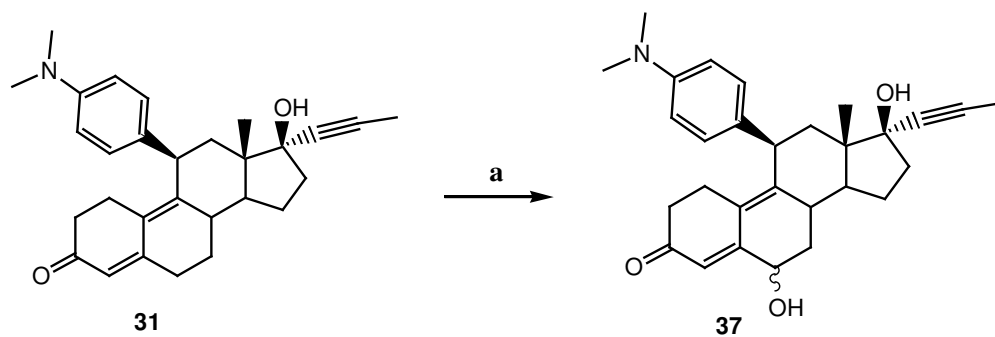
(a) 5 equiv. TMSCl, THF, 80%

Because two new stereocenters have been generated with no control in this reaction, four diastereomers were produced. The Bhc-diol-mifepristone was mixed in KMOPS buffer and photolyzed under a 365 nm UV lamp, but there was no change in concentration even after 10 min. One of the possibilities that might cause a photolysis problem is the low solubility of the Bhc-diol-mifepristone in aqueous solution. Several different conditions were tried: HEPES buffer (pH = 7.4) was used instead of KMOPS buffer; a large amount of an organic solvent, such as acetonitrile, methanol, or DMSO was used to increase its solubility in aqueous buffer. The best condition was when 200 μL of DMSO were added to dissolve the Bhc-diol-mifepristone in 3 mL KMOPS buffer. The photolysis under UV light (365 nm) still took very long under these conditions (Figure 12). It is impractical for biological systems. The other possibility for the photolysis problem is that Bhc excitation might be quenched by the aniline moiety of mifepristone. Mifepristone analogs, also capable of inducing expression, might alleviate this problem.

Figure 12. The Time Courses of One-photon Photolysis of Bhc-diol-mifepristone
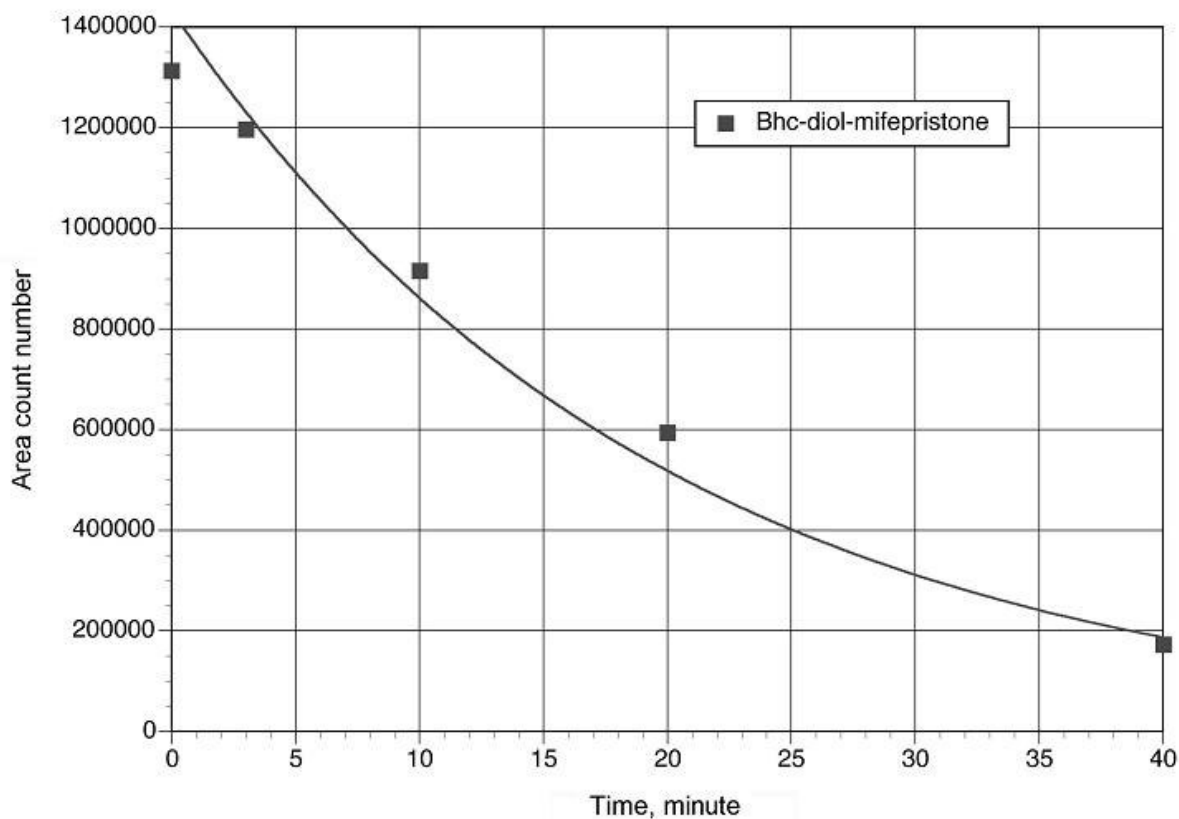

A modification is to make the trimethylammonium salt of Bhc-diol-mefipristone and to test its photochemistry. We will make trimethylammonium salts of Bhc-diol-mifepristone (**34**) by using CH_3I in diethyl ether with Bhc-diol-mifepristone (**33**) (Scheme 6). The ^1H NMR and mass spectrum showed that the product obtained under this condition was the trimethylammonium salt of Bhc-diol-mefipristone, **34**. Further characterization will be done to confirm its formation. The ammonium salt might increase the solubility of the caged compounds and change the electronic distribution of the compound in a way that will facilitate the photolysis of the messenger.

Analogs of mifepristone might work for the same purpose in the gene expression system. One alternative (**35**, desmethylmifepristone) has been synthesized in our lab and coupled to BhcOH (Figure 13) but surprisingly, the carbamate caged compound (**36**, Bhc-

desmethylmifepristone) cannot mask the messenger's physiological activity. Another possible alternative is hydroxymifepristone **36**[85] (Scheme 7). The hydroxymifepristone will be also coupled with BhcOH as a carbonate (**37**) (Scheme 8). The hydroxymifepristone can also be modified by amination. As a precursor of carbamate **41**, intermediate **39** has been made by a Mitsunobu reaction by using diisopropyl azodicarboxylate (DIAD), phthalimide, and triphenylphosphine (Scheme 9).

Scheme 6. Formation of Trimethyl Ammonium Salts of Bhc-diol-mifepristone

33   **34**

(a) CH₃I, (CH₃CH₂)₂O

Figure 13. Desmethylmifepristone and Bhc-desmethylmifepristone

35 : Desmethylmifepristone   **36** : Bhc-Desmethylmifepristone

Scheme 7. Synthesis of (6α- and 6β-)hydroxymifepristone

(a) SeO$_2$, dioxane, 39%.

Scheme 8. Synthesis of Bhc-mifepristone carbonate
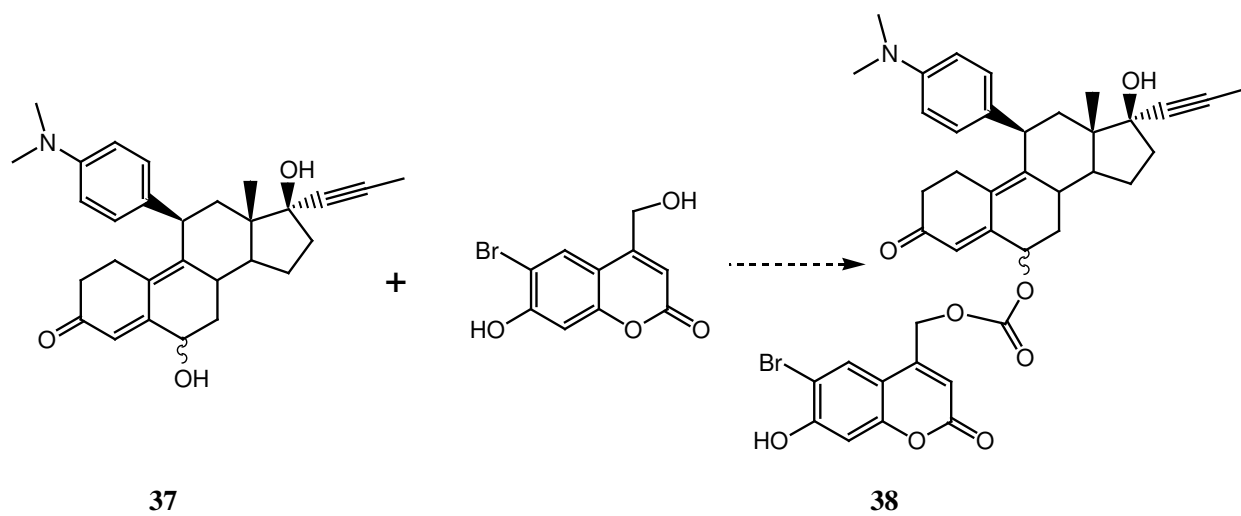
Scheme 9. Synthesis of Bhc-mifepristone carbamate.

37   **a**   **39**   **40**

41
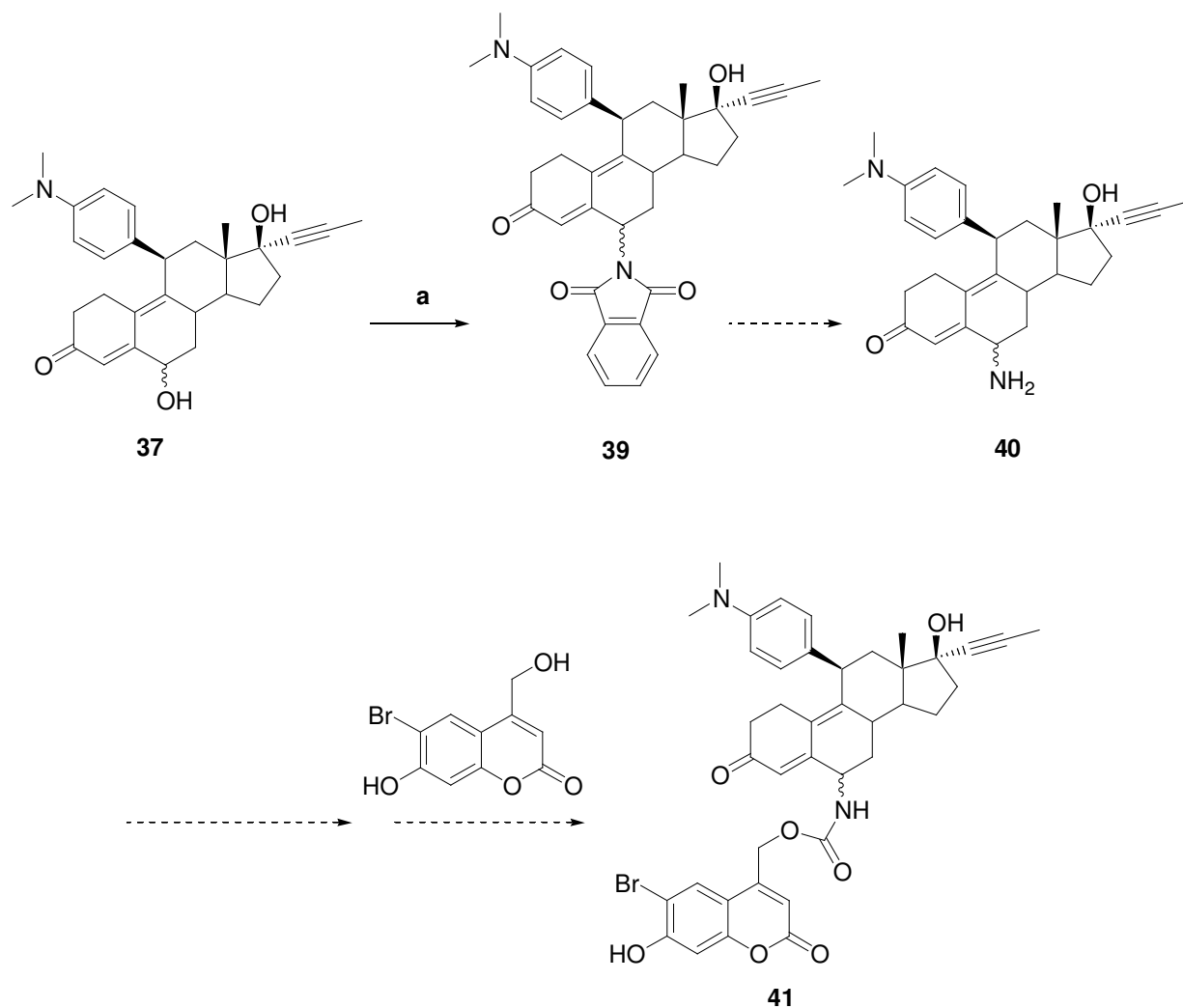
(a) DIAD, phthalimide, THF, Ph$_3$P.

The synthesis and application of a new photoremovable protecting group Bhc-diol has been described in this thesis. The presented data and results indicated Bhc-diol possesses sufficient one-photon quantum efficiency and two-photon uncaging cross-section for this purpose, and it is comparable to other known coumarin derivatives. The successful use of caged

compounds in cellular studies requires solubility in aqueous solutions and hydrolytic stability in the absence of light. Our Bhc-diol-caged aldehydes and ketones have good solubility in water and they are hydrolytically stable in the dark. The data we have collected shows that Bhc-diol has the ability to be used as a photoremovable protecting group to cage aldehydes and ketones and the promise of Bhc-diol as a good photo-mediator of drug delivery. Upon photolysis, the Bhc-diol caged aldehydes and ketones can release free carbonyl compounds under simulated physiological conditions. Although there were some problems to photolyze Bhc-diol-mifepristone efficiently, Bhc-diol still has the potential to cage other biologically active messengers containing carbonyl functional groups. After synthesizing the desired Bhc-diol-mifepristone (**32,** without conjugated double bonds migration), solving the problem involved in photolysis of Bhc-diol-mifepristone, or developing Bhc caged mifepristone analogs, a practical light-switchable gene expression system could be created. Since Bhc has been widely used in biological systems, Bhc-diol is the first example of a photoremovable protecting group capable of releasing aldehydes and ketones by single- and two-photon excitation under physiological conditions. Further investigation into the mechanism of photolysis and utility in biological systems is underway.

CHAPTER 3

EXPERIMENTAL SECTION

All reagents and solvents were purchased from commercial sources and used without further purification with the following exceptions. Toluene was dried by passing it through activated alumina under nitrogen pressure (Solv-Tek, Berryville, VA). Pyridine and acetonitrile were refluxed with calcium hydride under nitrogen, and then distilled. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Mercury Plus 400 MHz spectrometer using tetramethylsilane (TMS) as an internal standard. Chemical shift data for the proton resonances were reported in parts per million (δ) relative to internal standard TMS (δ 0.00). Chromatographic solvent proportions are expressed on a volume: volume basis. FTIR spectra were recorded on a Brucker Vector 22 spectrophotometer. UV spectra were recorded on a Cary 300 Bio UV-Visible spectrophotometer (Varian). HPLC analysis (analytical and preparative) was performed on a Varian ProStar HPLC system with an autosampler and diode array detector using Microsorb C-18 reverse phase columns. Mass spectrometry was performed on a Sciex API-1 Plus quadrupole mass spectrometer with an electrospray ionization source. KMOPS buffer consisted of 100 mM KCl and 10 mM MOPS titrated to pH 7.2 with KOH. Thin layer and column chromatography were performed on precoated silica gel 60 F_{254} plates (EM Science) and 230-400 mesh silica gel 60 (EM Science), respectively. Melting points were determined on a Mel-Temp (Laboratory Devices, Inc.), and are uncorrected.

## 6-BROMO-4-CHLOROMETHYL-7-ACETYLOXYCOUMARIN (26).

85%
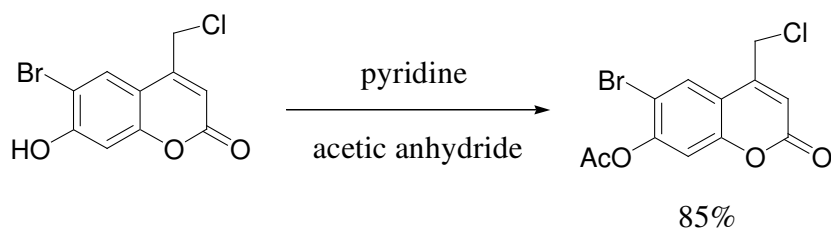
Under a nitrogen atmosphere, 6-bromo-4-chloromethyl-7-hydroxycoumarin (780 mg, 2.69 mmol) and acetic anhydride (0.64 ml, 6.75 mmol) were stirred in anhydrous pyridine (3 ml) for 4 h. The pyridine was removed under vacuum, and the resulting crude residue was purified by flash chromatography through silica gel (hexane/ethyl acetate 1:1) to afford a white solid (760 mg, 85%, mp 180 °C dec). ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.91 (1H, s), 7.21 (1H, s), 6.58 (1H, s), 4.63 (2H, s), 2.41 (3H, s); ^{13}C NMR (100 MHz, CDCl$_3$) 168.06, 159.45, 153.68, 150.99, 148.31, 128.61, 116.96, 113.30, 112.40, 41.19, 21.03; FTIR (neat) 1732, 1599, 1396, 1217, 1181, 1137, 1022, 906, 862, 728, 658, 629 cm^{-1}.
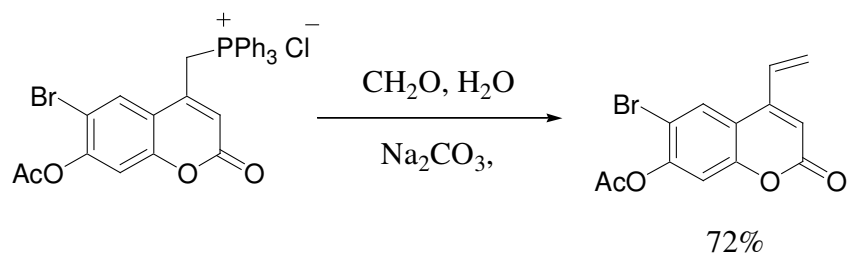
PHOSPHONIUM SALT (27).

82%
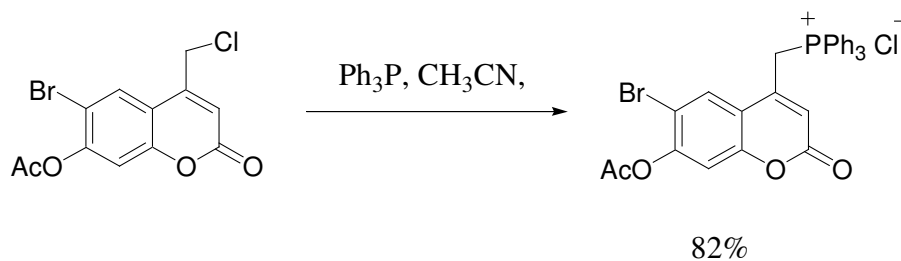
Under a nitrogen atmosphere, a mixture of 6-bromo-4-chloromethyl-7-acetyloxycoumarin (650 mg, 1.96 mmol) and triphenyl phosphine (1.028 g, 3.92 mmol) in acetonitrile (5 ml) was heated to 85 °C. After 30 min, the suspended mixture dissolved. Heating

was continued for 18 h, during which period the white phosphonium salt precipitated. The mixture was cooled, filtered and the filtrand was washed several times with boiling benzene to yield a white solid (948 mg, 82%), which was carried to the next step without further purification.
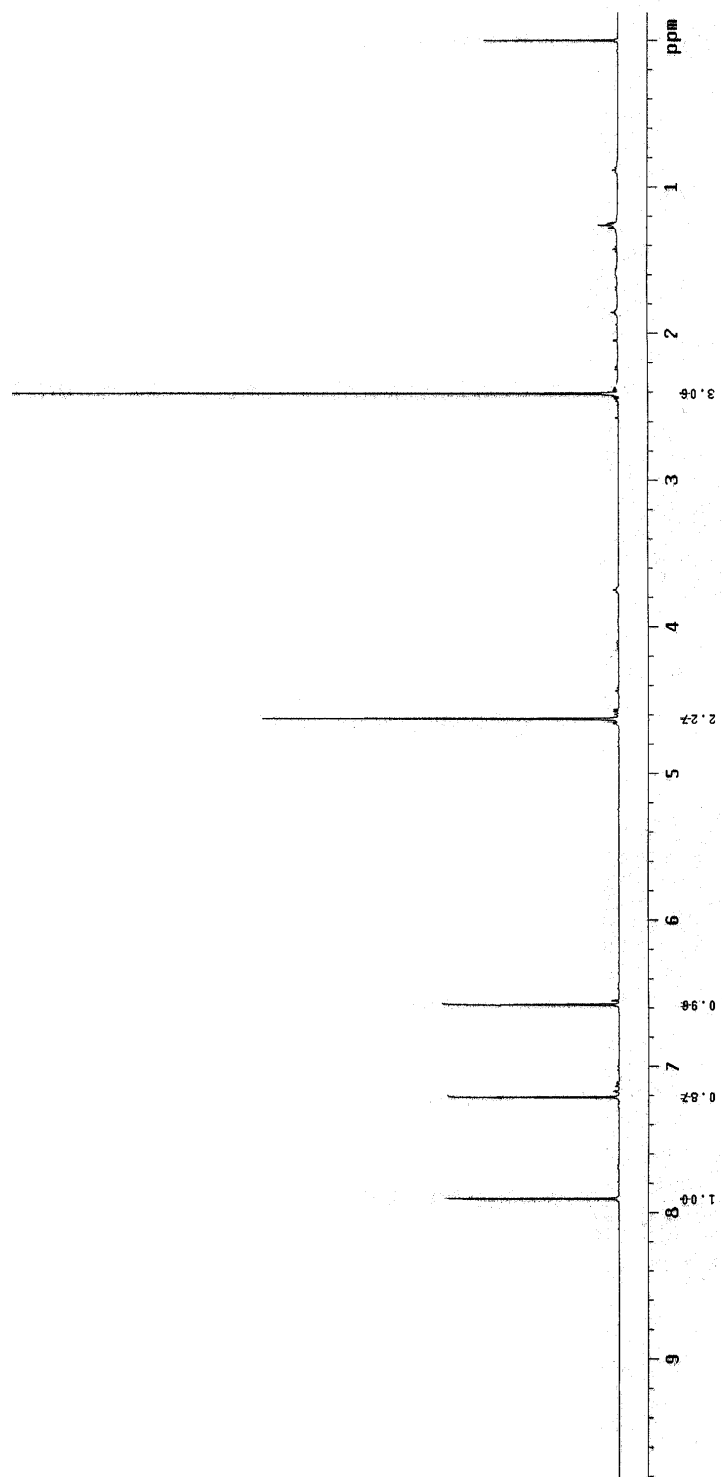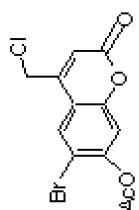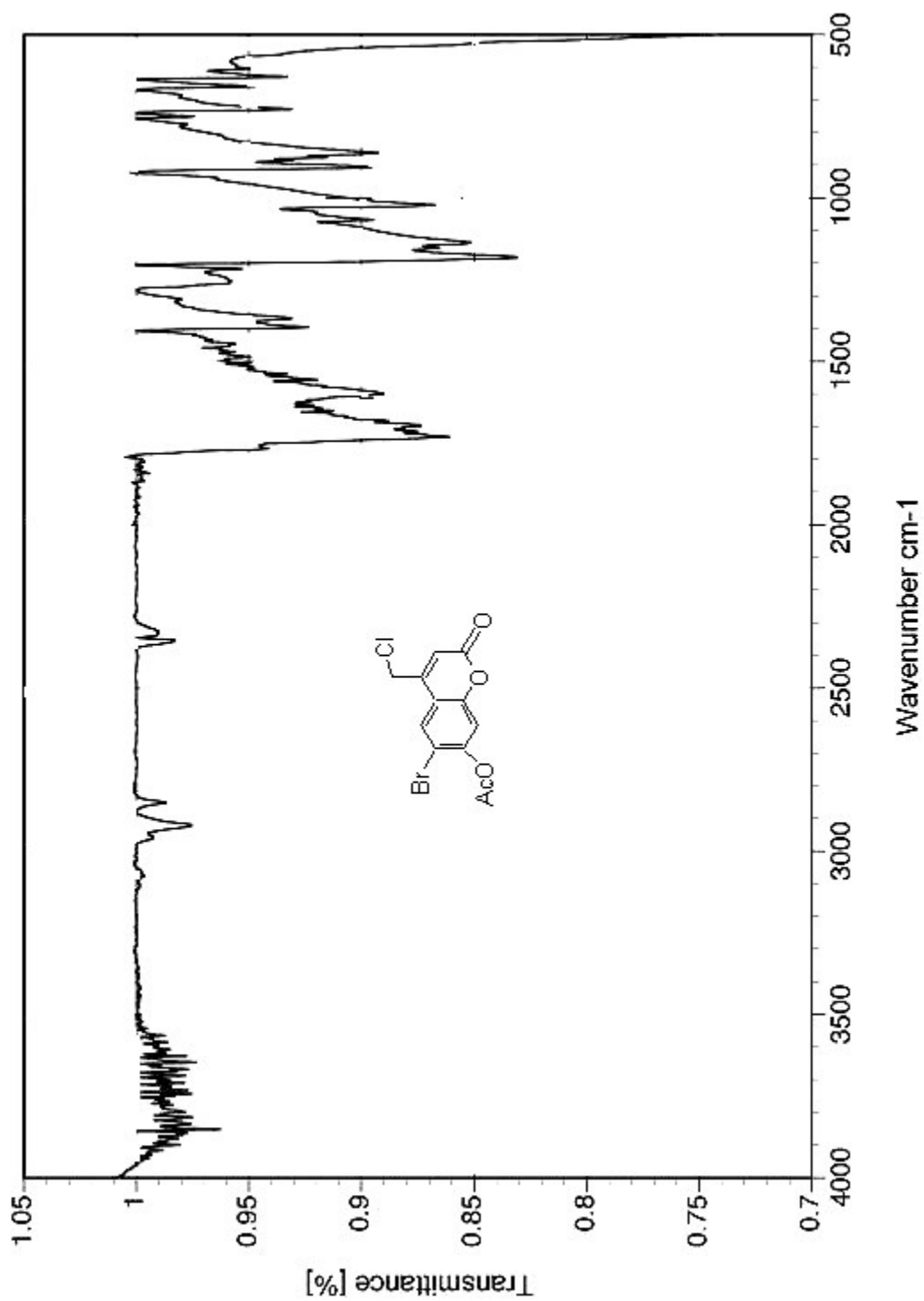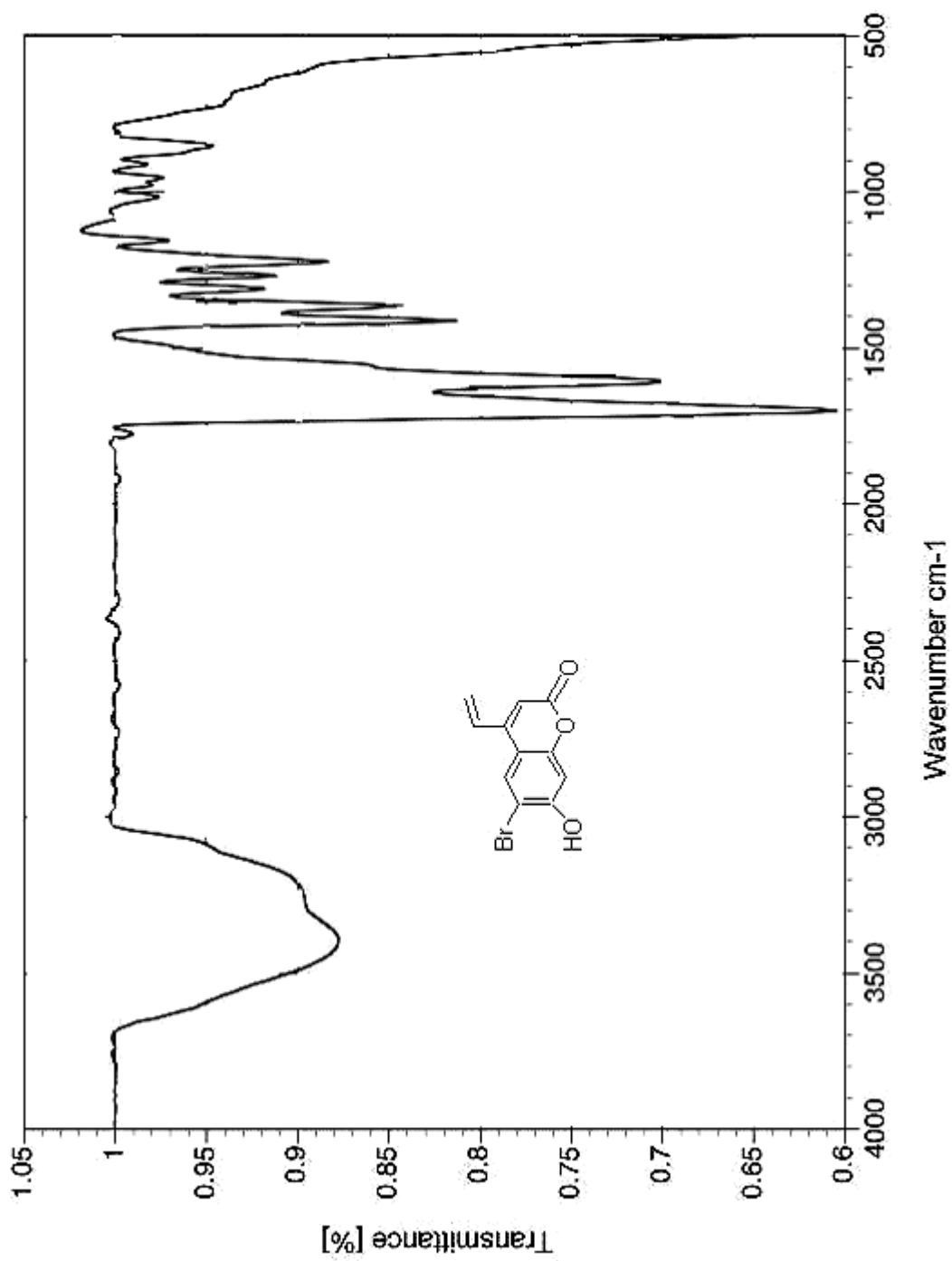
**6-BROMO-7-HYDROXY-4-VINYLCOUMARIN(BHC-VINYL) (28).**

72%

Phosphonium salt (310 mg, 0.522 mmol) was taken up in a 37% formaldehyde solution (4 ml, aqueous). The mixture was stirred for 15 min, and then an aqueous solution of 15% Na_2CO_3 (0.5 ml) was added intermittently by a syringe. Each subsequent addition was made after the orange-yellow color of the phosphorone formed had disappeared. When the addition of the base was complete, the mixture was stirred at room temperature for 2 h, and then extracted three times with chloroform. The combined chloroform extracts were dried over anhydrous sodium sulfate and evaporated. The crude product was purified by flash chromatography through silica gel (hexane/ethyl acetate 6:4) to afford a white solid (100 mg, 72%, mp 210-230 °C dec). ^{1}HNMR (400 MHz, $(CD_3)_2CO$) δ 8.00 (1H, s), 7.21 (1H, dd, J = 17.2, 11.6 Hz), 6.94 (1H, s), 6.35 (1H, s), 6.14 (1H, dd, J = 17.2, 1.2 Hz), 5.77 (1H, dd, J = 11.2, 0.8 Hz); ^{13}C NMR (100 MHz, $(CD_3)_2CO$) 160.08, 157.31, 154.88, 150.18, 130.47, 129.19, 123.31, 112.80, 108.38, 106.08, 103.87; FTIR (neat) 3392, 1700, 1607, 1412, 1365, 1310, 1268, 1223, 1155, 954 cm^{-1}; MS m/z 267 (MH[+], [81]Br), 265 (MH[+], [79]Br).
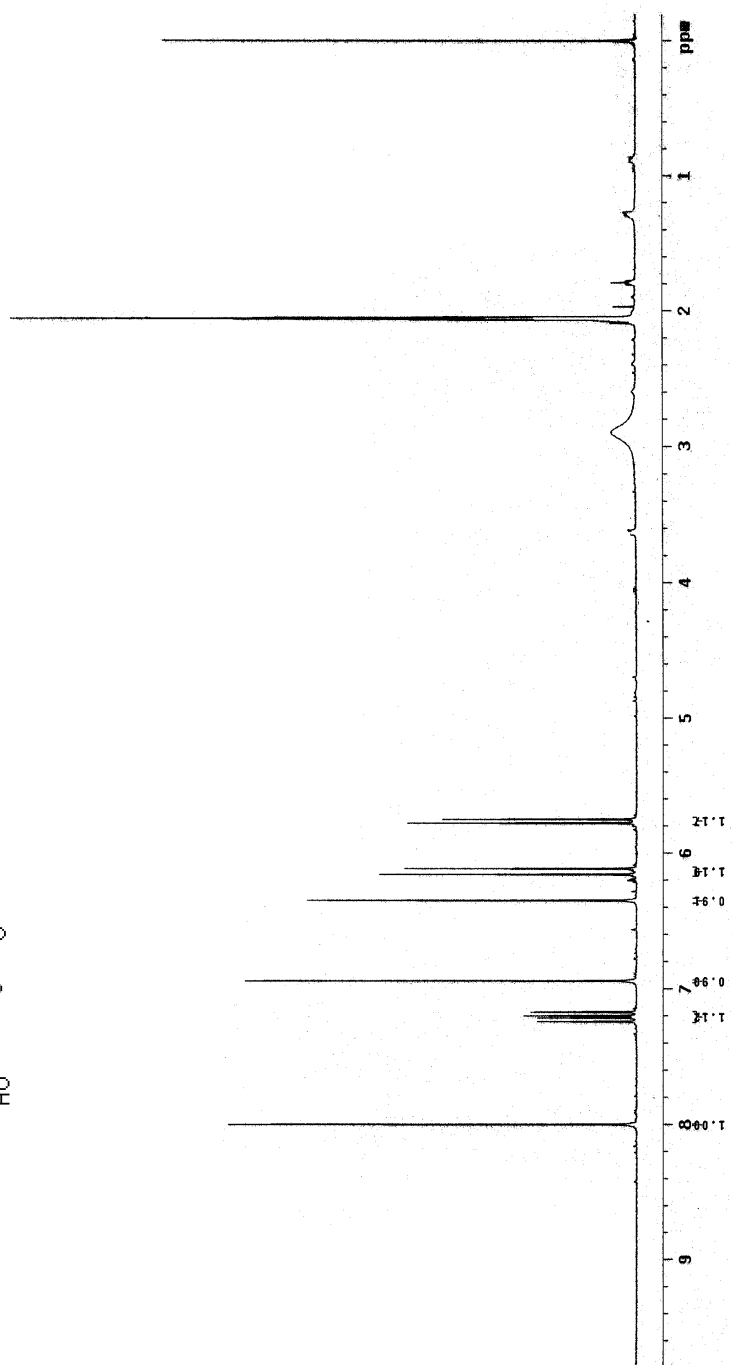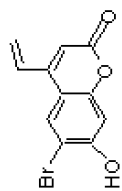
6-BROMO-4-(1,2-DIHYDROXYETHYL)-7-HYDROXYCOUMARIN(BHC-DIOL) (20).

80%
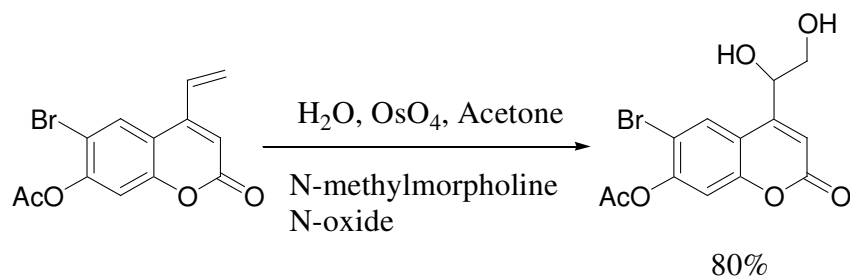
Bhc-vinyl (200 mg, 0.749 mmol) and one small piece of osmium tetroxide (which was not weighed because of its toxicity and the difficulty in accurately weighing it, due to rapid sublimation) were added to a solution of 4-methylmorpholine N-oxide monohydrate (101 mg, 0.747 mmol) in water (4 ml) and acetone (2 ml). The resulting solution was vigorously stirred at room temperature for 18 h, during which time the mixture turned a light brown color. The reaction was neutralized to pH 7 with 3 N sulfuric acid. The acetone was evaporated, and the resulting aqueous solution was extracted five times by ethyl acetate. The combined organic extracts were evaporated, and the resulting crude product was purified by flash chromatography through silica gel (hexane/ethyl acetate 2:8) to afford a white solid (180 mg, 80%, mp 200-220 $^{\circ}$C dec). ^{1}HNMR (400 MHz, (CD$_3$)$_2$CO) δ 8.05 (1H, s), 6.92 (1H, s), 6.41 (1H, s), 5.13 (1H, dd, J = 4.4, 4.4 Hz), 3.88 (1H, dd, J = 12.0, 4.4 Hz), 3.71 (1H, dd, J = 11.2, 5.6 Hz); ^{13}C NMR (100 MHz, (CD$_3$)$_2$CO) 160.78, 157.55, 156.15, 155.57, 129.94, 113.09, 111.09, 106.56, 104.44, 71.42, 67.03; FTIR (neat) 3332, 1726, 1604, 1437, 1158, 1120, 882, 773, 695, 540 cm-1; MS m/z 303 (MH$^+$, ^{81}Br), 301 (MH$^+$, ^{79}Br).
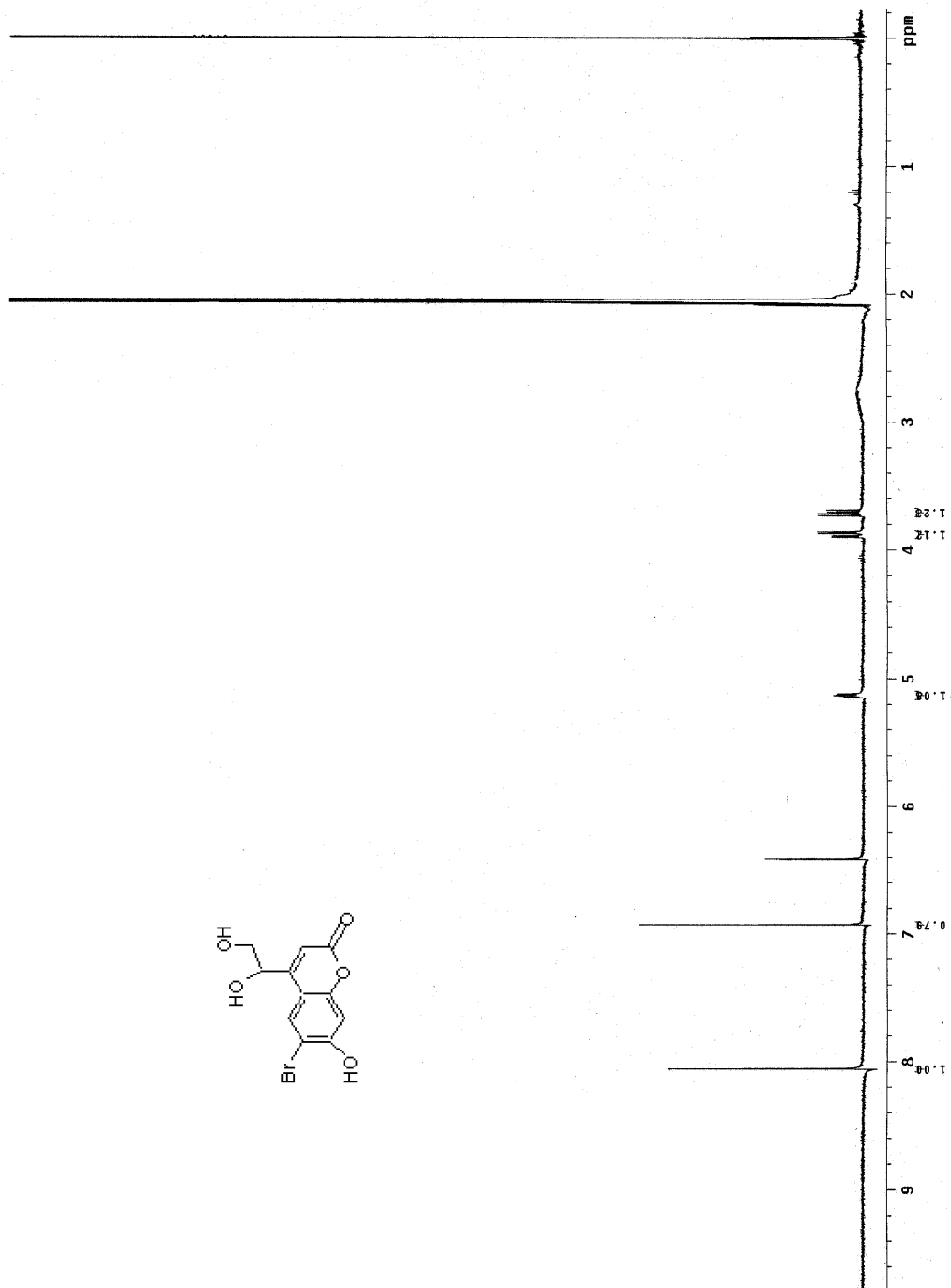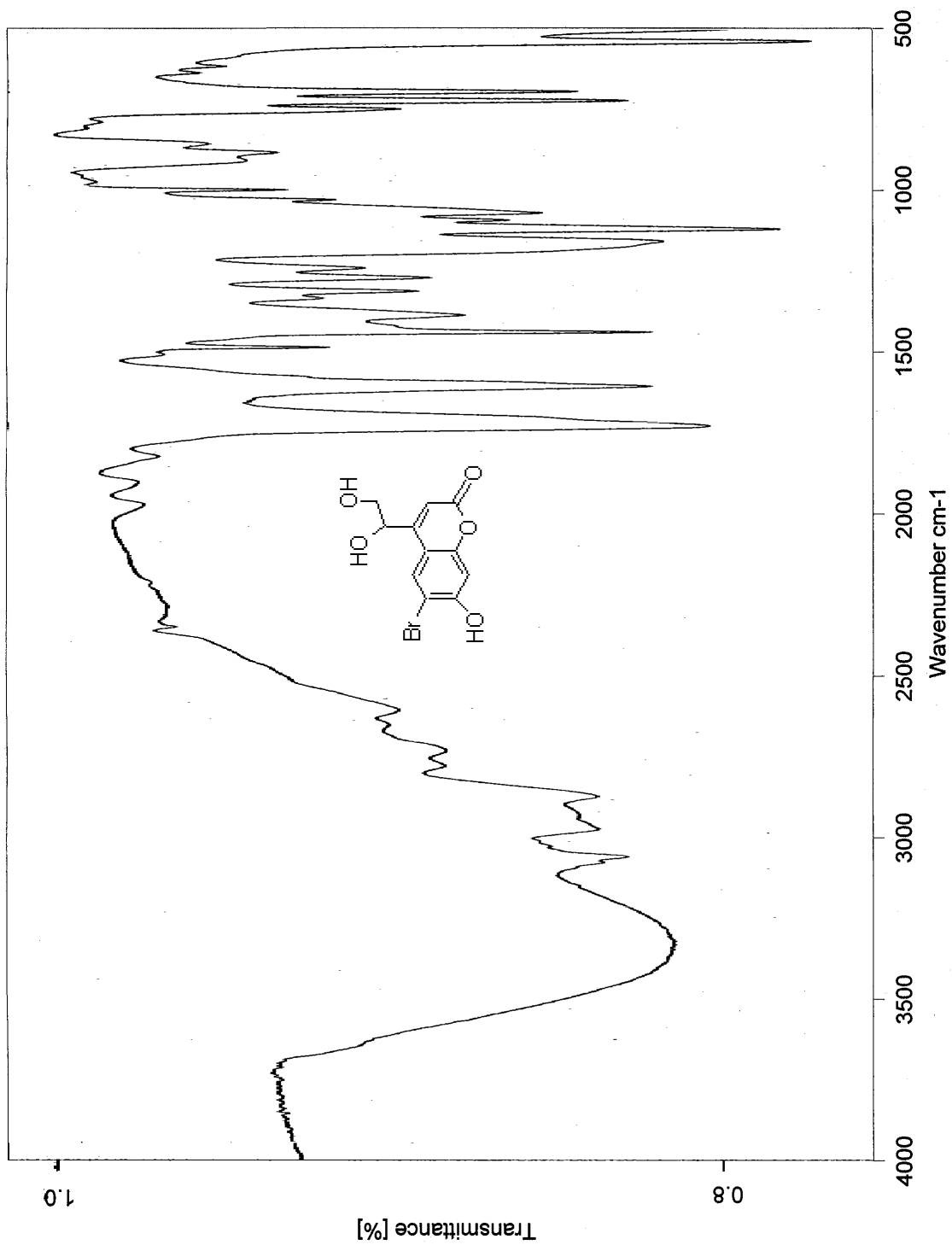
GENERAL PROCEDURE FOR THE SYNTHESIS OF 6-BROMO-7-HYDROXYCOUMARIN ACETALS AND KETALS.

Under a nitrogen atmosphere, aldehyde or a ketone (2 equiv.) in 10 μl of 1-butanol was added to a mixture of Bhc-diol (1 equiv.), pyridinium p-toluene sulfonate (PPTS, 1 equiv.), and anhydrous MgSO$_4$ (100 mg) in anhydrous toluene (2 ml). The reaction mixture was stirred at 110 °C for 8 h, and then filtered, washing the solid filtrand with chloroform. The filtrate was evaporated, and the resulting crude product was purified by flash chromatography through silica gel (ethyl acetate/hexanes 1:1) to give the Bhc-protected aldehyde or ketone. With the exception of Bhc-diol-cyclohexanone, all Bhc-diol protected aldehydes and ketones were isolated as a mixture of diastereomers.

BHC-DIOL-BENZALDEHYDE (30a).
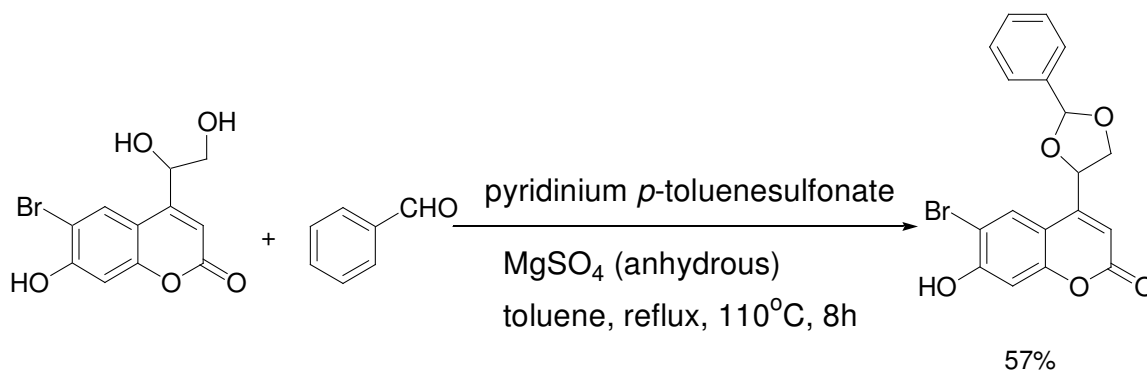
Bhc-diol (15 mg, 0.05 mmol), PPTS (13 mg, 0.05 mmol), benzaldehyde (10 μl, 0.10 mmol); yield = 11 mg (57%, mp 106-180 °C dec). ^{1}HNMR (400 MHz, CDCl$_3$) δ 7.50 (6H, m), [7.07 (s), 7.06 (s) (1H)], [6.67 (s), 6.55 (s) (1H)], [6.14 (s), 6.02 (s) (1H)], 5.40 (1H, m), [4.73 (dd, J = 7.6, 7.6 Hz), 4.57 (dd, J = 8.4, 8.4 Hz) (1H)], [4.09 (dd, J = 8.4, 5.6 Hz), 3.86 (dd, J =

7.2, 7.2 Hz) (1H)]; ^{13}C NMR (100 MHz, CDCl$_3$) (160.44, 160.33), (155.37, 155.27), (154.91, 154.85), (152.14, 151.85), 136.57, 135.58, (129.96, 129.76), (128.68, 128.62), 126.67, (126.54, 126.44), (112.24, 112.15), 110.70, 109.60, 106.68, 105.18, (104.74, 104.67), (73.80, 73.30), (70.62, 70.23); FTIR (neat) 3407, 2922, 1698, 1602, 1396, 1308, 1271, 1222, 1153, 1095, 1021, 873, 756, 699 cm^{-1}; MS *m/z* 391 (MH$^+$, ^{81}Br), 389 (MH$^+$, ^{79}Br).
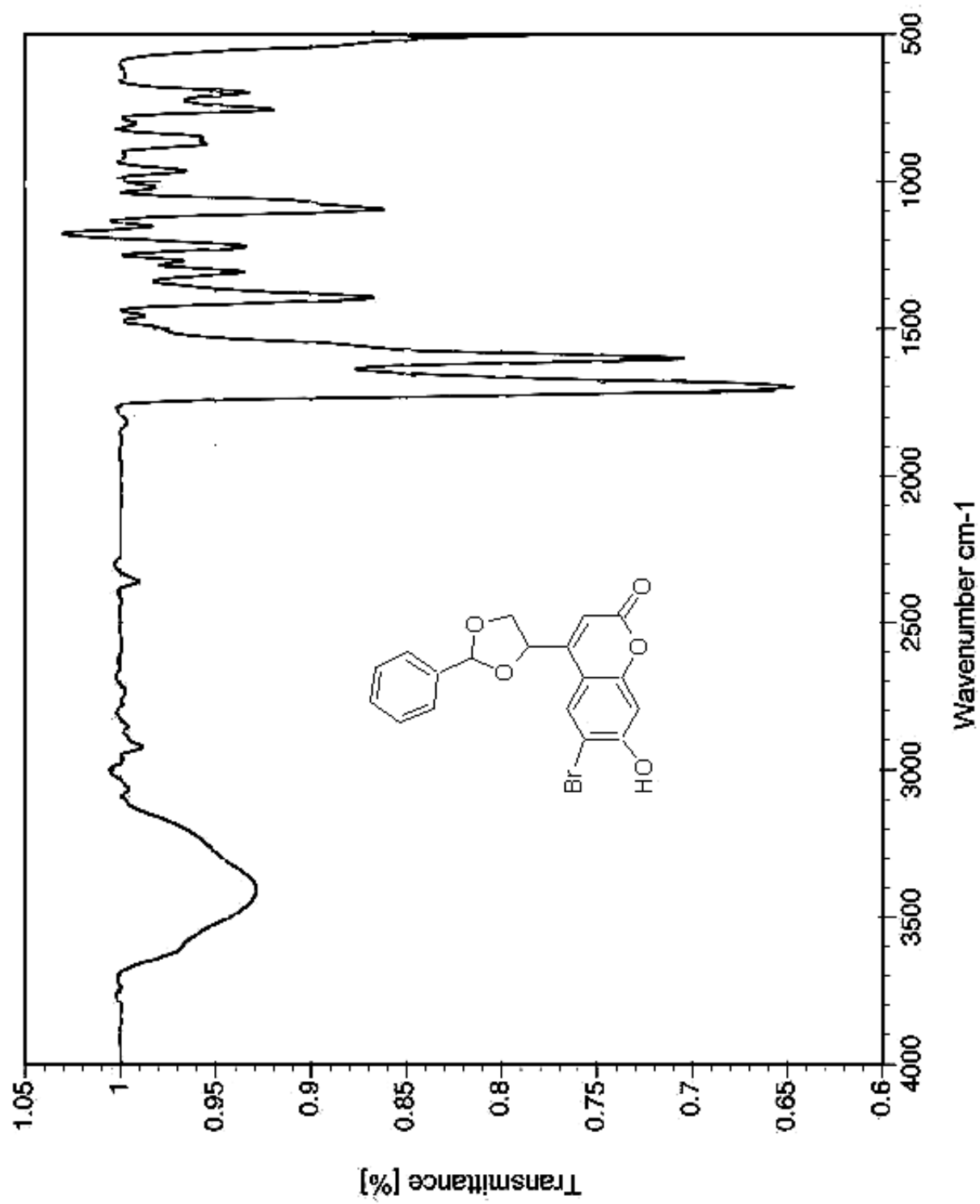
BHC-DIOL-PIPERONAL (30b).
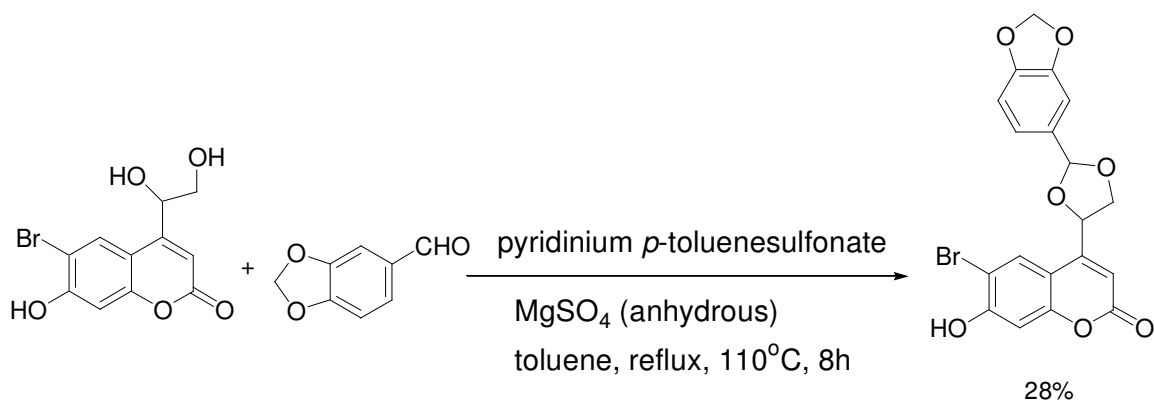
Bhc-diol (15 mg, 0.05 mmol), PPTS (13 mg, 0.05 mmol), piperonal (15 mg, 0.10 mmol); yield = 6 mg (28%, mp 176-195 $^{\circ}$C dec). ^{1}HNMR (400 MHz, CDCl$_3$) δ [7.58 (s), 7.50 (s) (1H)], 7.06 (3H, m), [6.87 (s), 6.85 (s) (1H)], [6.64 (s), 6.54 (s) (1H)], 6.02 (2H, m), [6.02 (s), 5.92 (1H)], [5.40 (dd, J = 6.8, 6.8 Hz), 5.35 (dd, J = 6.8, 5.6 Hz) (1H)], [4.74 (dd, J = 7.6, 7.6 Hz), 4.54 (dd, J = 8.0, 8.0 Hz) (1H)], [4.07 (dd, J = 7.6, 5.2 Hz), 3.82 (dd, J = 7.6, 7.6 Hz) (1H)]; ^{13}C NMR (100 MHz, CDCl$_3$) (160.38, 160.27), (155.34, 155.25), (154.96, 154.90), (152.10, 151.77), (148.97, 148.84), 148.01, (130.44, 129.46), (126.70, 126.55), (120.96, 120.76), (112.28, 112.20), (110.74, 109.61), (108.29, 108.22), 106.92, (106.72, 106.69), (105.05, 104.65), (104.76, 104.70), (101.40, 101.30), (73.76, 73.28), (70.62, 70.12); FTIR (neat) 3423, 2960, 2923, 1722, 1605, 1400, 1260, 1094, 1029, 869, 801 cm^{-1}; MS m/z 435 (MH$^+$, ^{81}Br), 433 (MH$^+$, ^{79}Br).
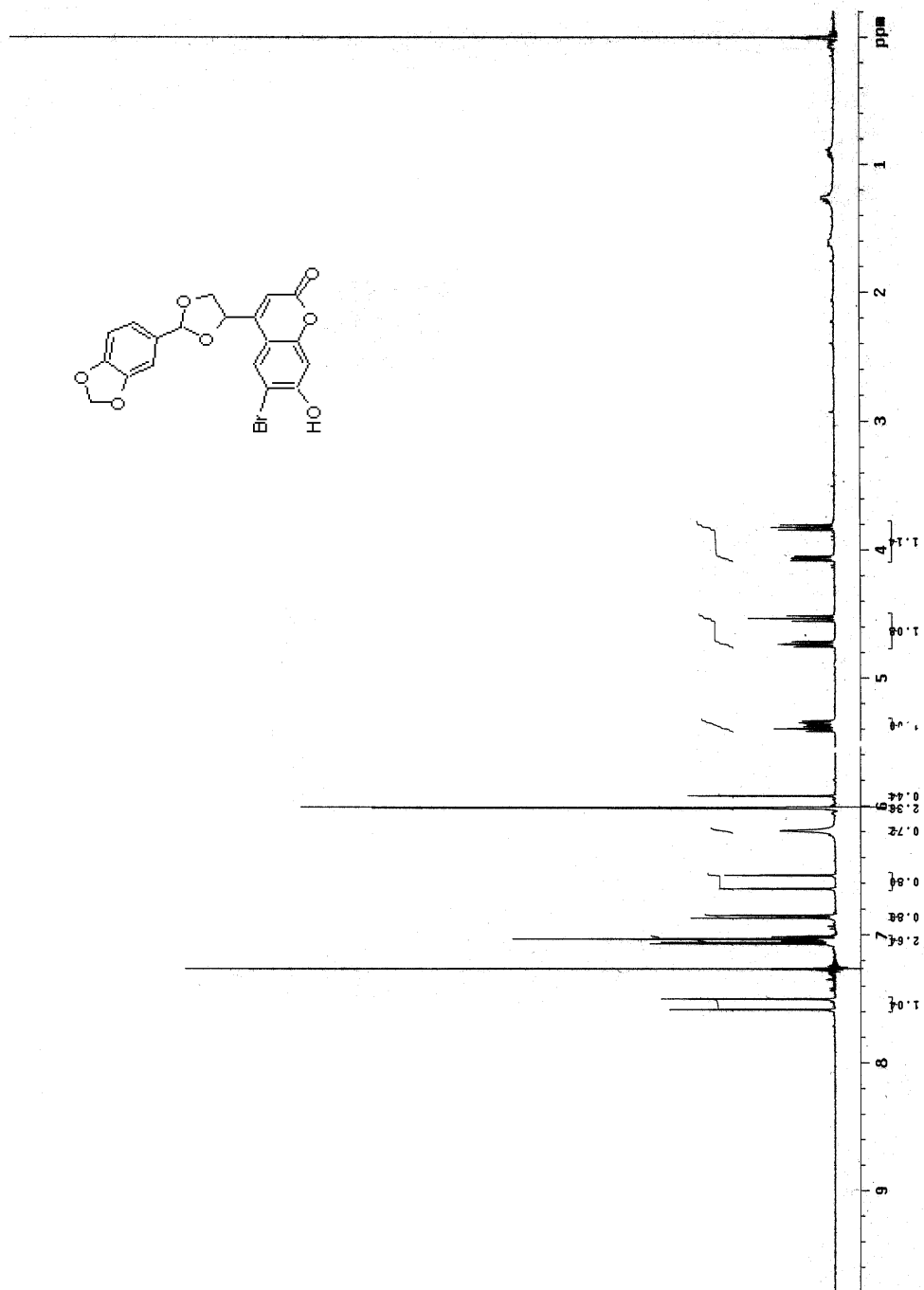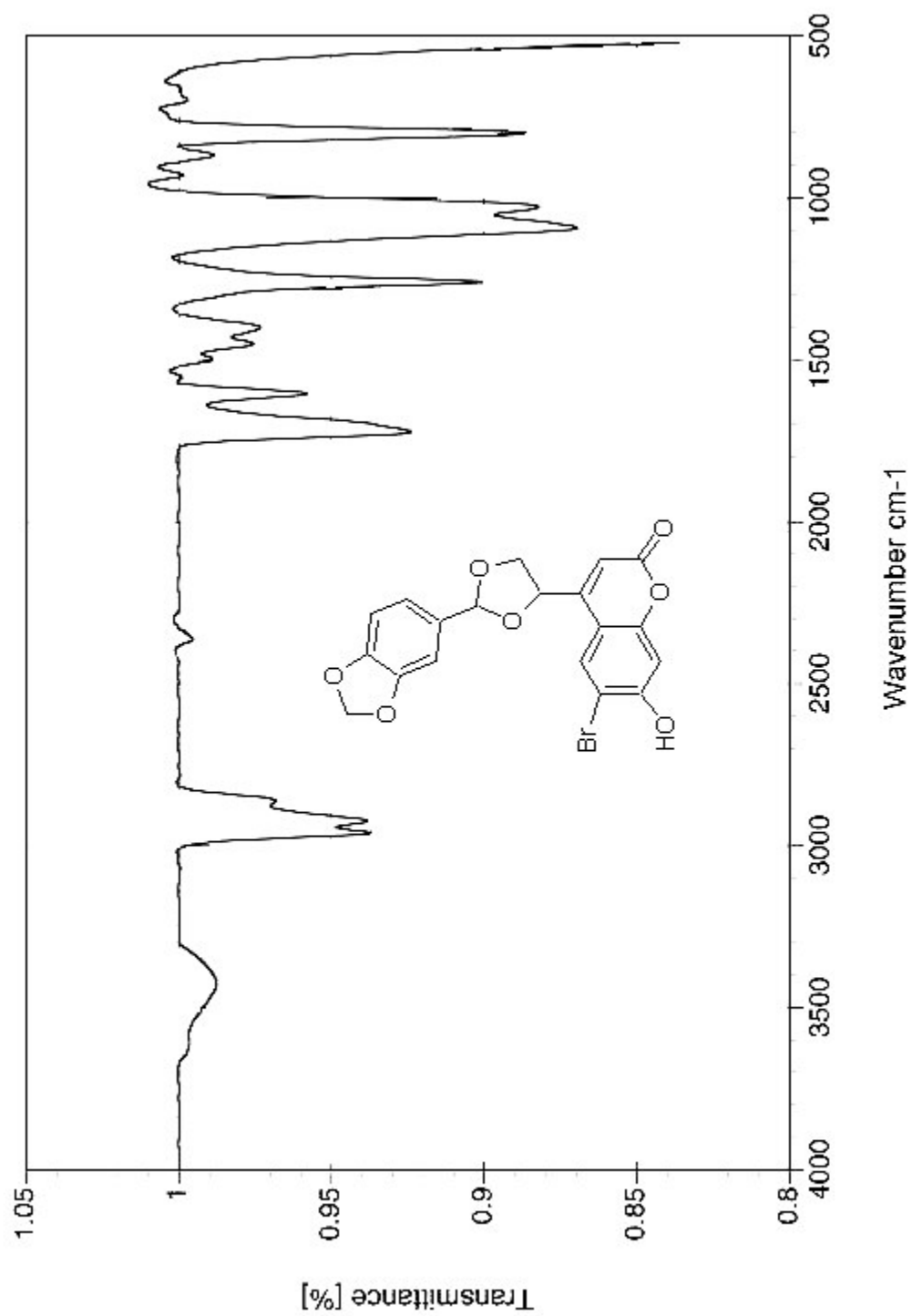
BHC-DIOL-ACETOPHENONE (30c).
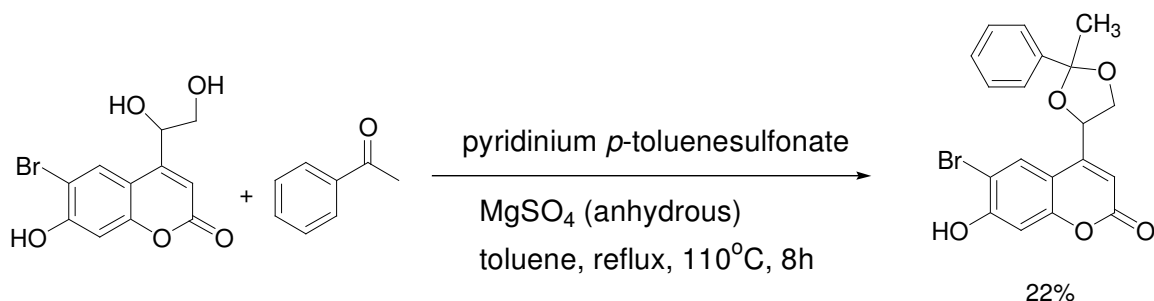
Bhc-diol (20 mg, 0.07 mmol), PPTS (33 mg, 0.14 mmol), acetophenone (40 μL, 0.35 mmol); yield = 6 mg (22%, mp 170-220 °C dec). ^{1}HNMR (400 MHz, CDCl$_3$) δ 7.51 (6H, m), [7.05 (s), 7.01 (s) (1H)], [6.64 (s), 6.40 (s) (1H)], [5.40 (dd, J = 7.2, 7.2 Hz), 5.07 (dd, J = 7.6, 5.2 Hz) (1H)], [4.65 (dd, J = 8.8, 6.8 Hz), 4.24 (dd, J = 8.0, 8.0 Hz) (1H)], [3.89 (dd, J = 8.0, 5.2 Hz), 3.71 (dd, J = 8.0, 8.0 Hz) (1H)], [1.83 (s), 1.78 (s) (3H)]; ^{13}C NMR (100 MHz, CDCl$_3$) 160.59, 155.40, (154.81, 154.63), 152.56, 141.98, (128.60, 128.53), (128.47, 128.34), 126.66, 125.27, 124.70, 112.15, 111.11, 110.20, 106.63, (104.61, 104.51), 74.41, 72.94, (69.86, 69.33), (28.16, 27.74); FTIR (neat) 3416, 2922, 1697, 1602, 1395, 1308, 1224, 1155, 1096, 874, 760, 699 cm^{-1}; MS m/z 405 (MH$^+$, [81]Br), 403 (MH$^+$, [79]Br).
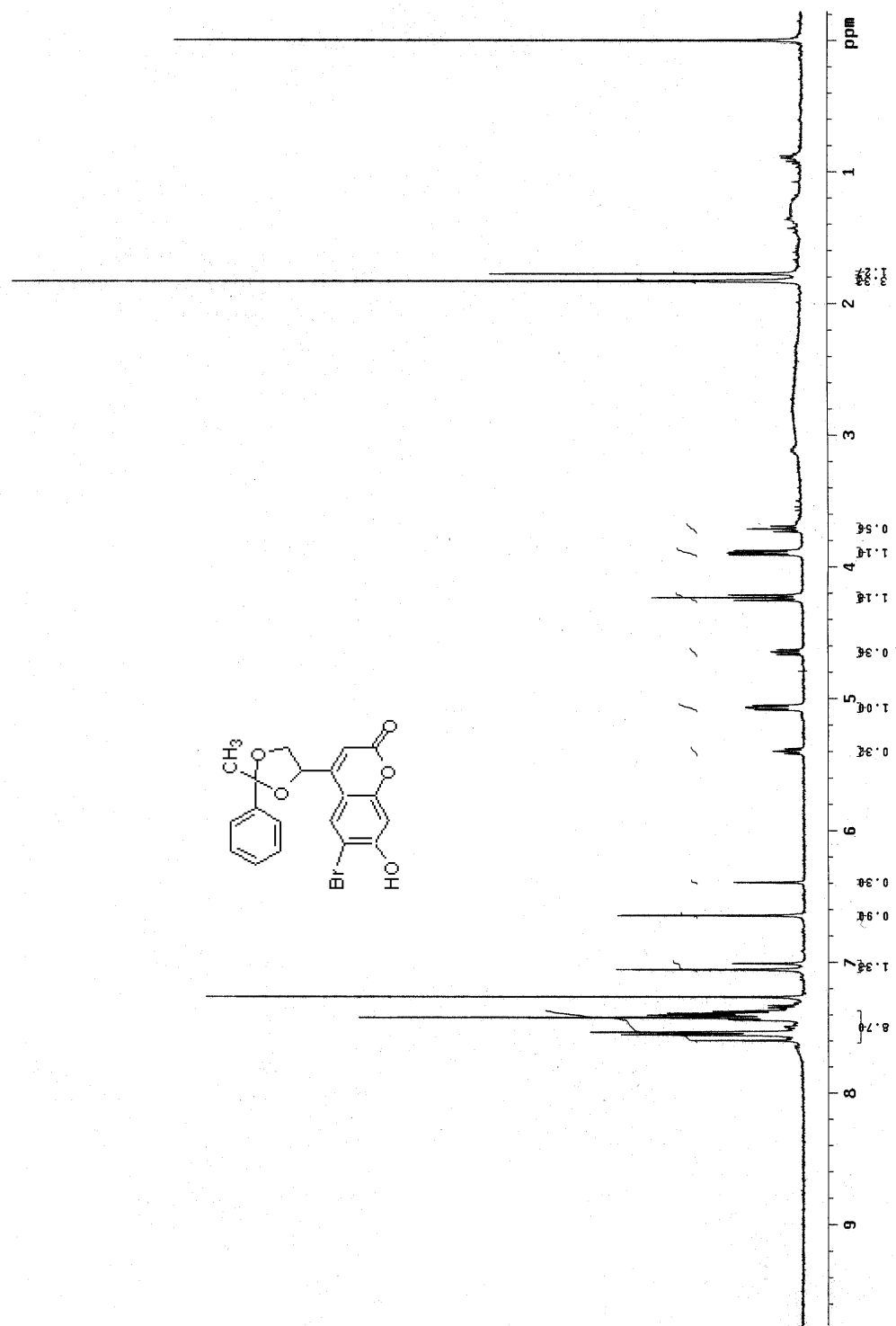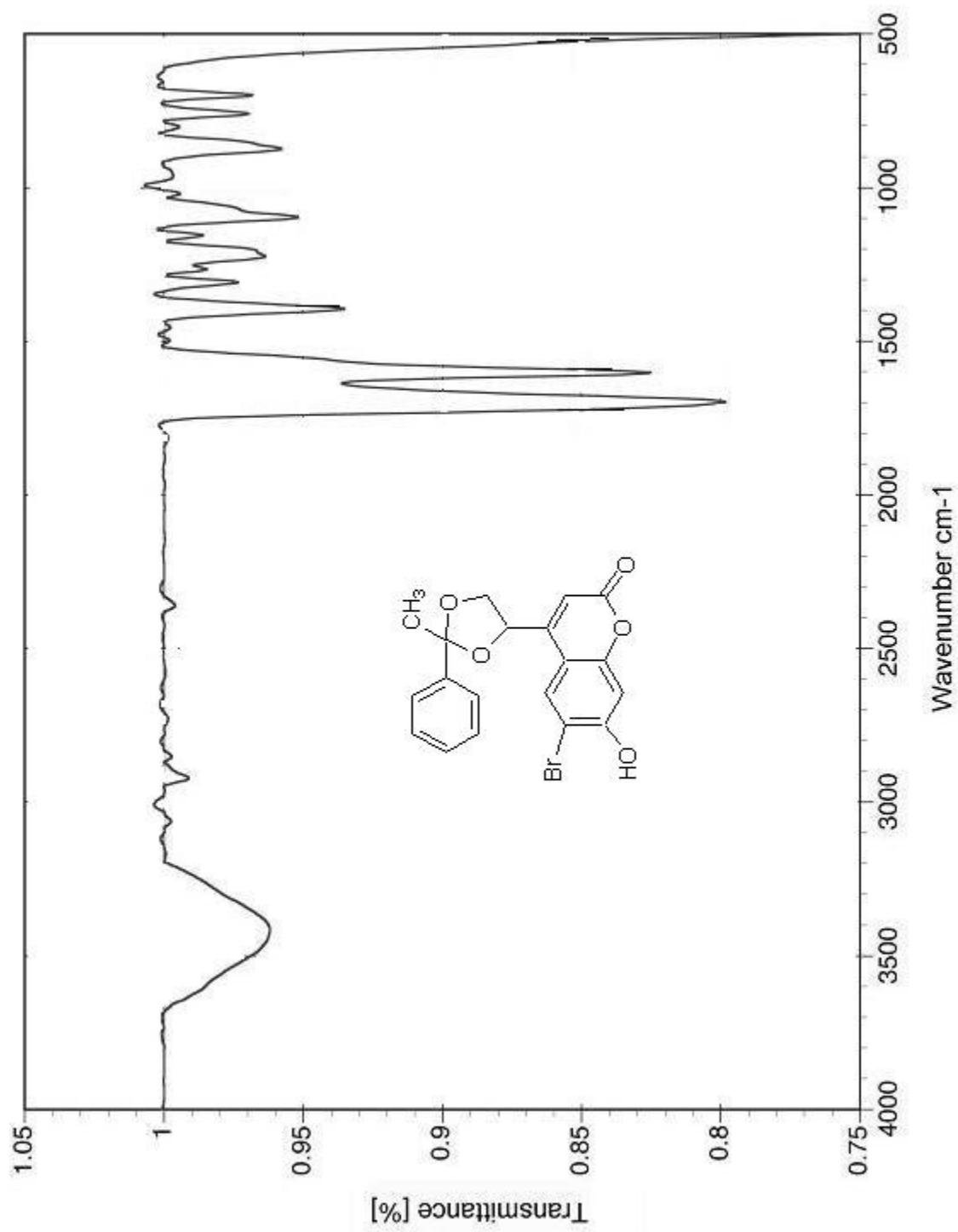
BHC-DIOL-CYCLOHEXANONE (30d).
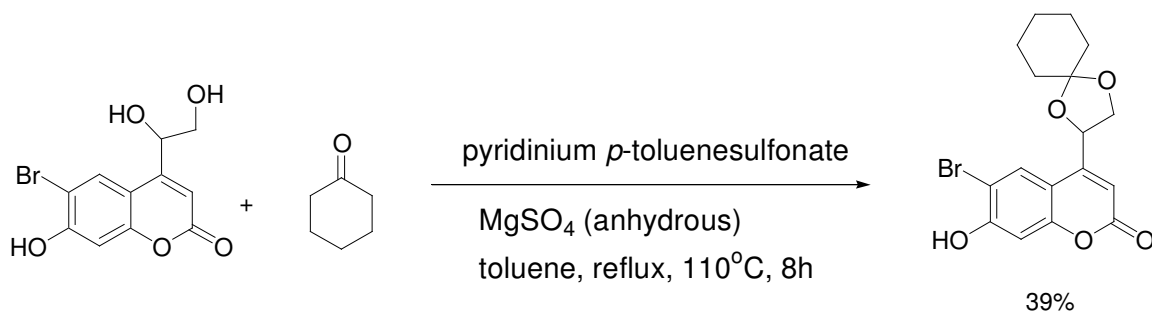
Bhc-diol (15 mg, 0.05 mmol), PPTS (13 mg, 0.05 mmol), cyclohexanone (10 μL, 0.10 mmol); yield = 7.5 mg (39%, mp 180-230 $^{\circ}$C dec). ^{1}HNMR (4500 MHz, CDCl$_3$) δ 7.54 (1H, s), 7.04 (1H, s), 6.58 (1H, s), 5.26 (1H, dd, J = 8.0, 8.0 Hz), 4.53 (1H, dd, J = 8.0, 8.0 Hz), 3.77 (1H, dd, J = 8.0, 8.0 Hz), 1.70 (10H, m); ^{13}C NMR (100 MHz, CDCl$_3$) 160.66, 155.26, 154.72, 152.69, 126.63, 112.38, 111.56, 109.89, 106.62, 104.60, 72.94, 69.09, 35.67, 34.81, 25.01, 23.89, 23.79; FTIR (neat) 3386, 2935, 2862, 1707, 1604, 1395, 1272, 1231, 1155, 1107, 1036, 875, 700 cm^{-1}; MS m/z 383 (MH$^+$, ^{81}Br), 381 (MH$^+$, ^{79}Br).

BHC-DIOL-MIFEPRISTONE (33).

80%

Mifepristone (50 mg, 0.12 mmol) is added to a solution of Bhc-diol (90 mg, 0.30 mmol) and dry THF (2 ml) under a nitrogen atmosphere. To the mixture is added chlorotrimethylsilane (75 µl, 0.58 mmol) and the mixture is stirred at reflux for 48 h. A saturated aqueous solution of sodium bicarbonate (5 ml) is added and the mixture extracted three times with ether, washed with brine, and the combined ether extracts were dried with anhydrous sodium sulfate and evaporated. The crude product was purified by flash chromatography on silica gel (hexane/ethyl acetate 4:6) to afford a yellow solid (68mg, 80%). ^{1}HNMR (400 MHz, CDCl$_3$) δ 7.51 (1H, m), 7.10 (2H, m), 7.02 (1H, m), 6.68 (2H, m), [6.57 (s), 6.55 (s), 6.49 (s), 6.41 (s) (1H)], 5.22 (1H, m), 4.49 (1H, m), 3.70 (1H, m), 2.95 (6H, m), 1.79 (3H, s), 0.97 (3H, s); FTIR (neat) 2919, 2357, 1718, 1604, 1516, 1395, 1351, 1308, 1268, 1215, 1150, 1113, 1091, 1062, 1012, 945, 876, 854, 818, 745, 665 cm^{-1}; MS *m/z* 714 (MH$^+$, ^{81}Br), 712 (MH$^+$, ^{79}Br).

%Transmittance

Wavenumbers (cm-1)
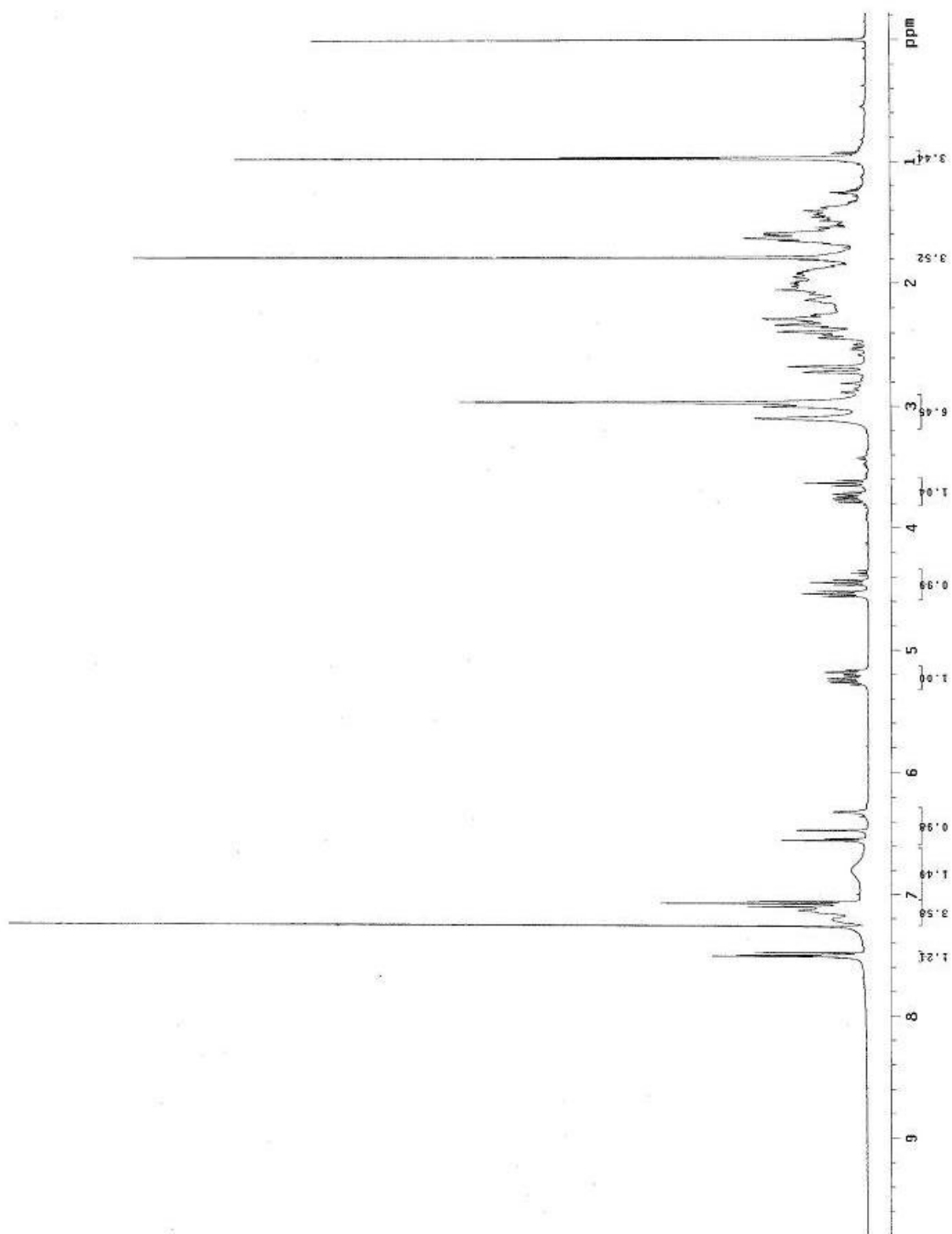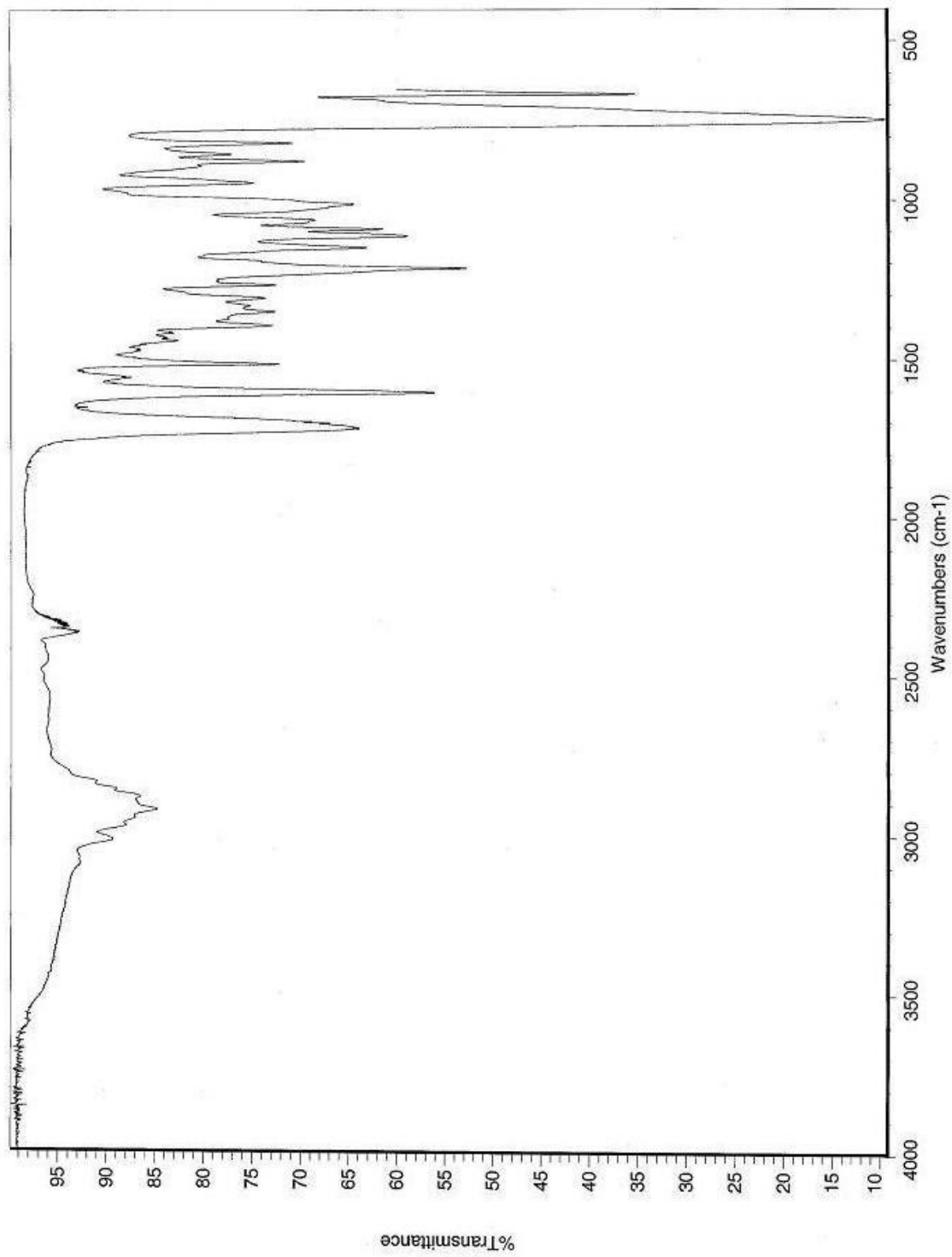
(6α- AND 6β-) HYDROXYMIFEPRISTONE (37).

39%
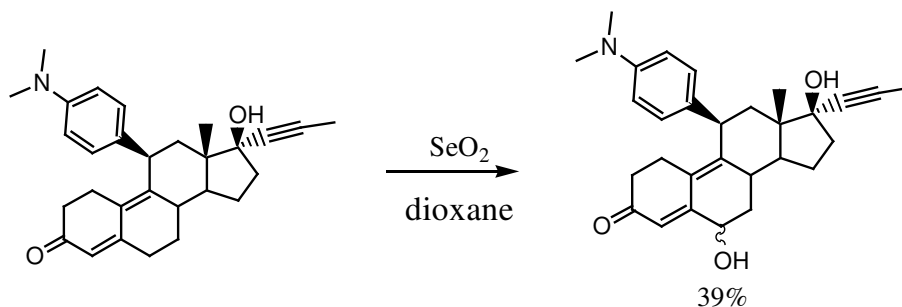
(11β,17β)-11-(4-Dimethylamino-phenyl)-17-hydroxyl-17-(1-propynyl)-estra-4,9-dien-3-one (mifepristone, RU486) (107 mg, 0.243 mmol) was dissolved in 50 ml dry dioxane in a 100 mL three-neck flask equipped with a reflux condenser. After addition of SeO_2 (33 mg, 0.303 mmol) the mixture was stirred at 80 °C under a N_2 atmosphere. The slightly yellow colored reaction mixture became red. The reaction was stopped after 20 h by addition of 30 mL 5% aqueous KOH solution. Thereafter, the solution was extracted four times with ethyl acetate, and the combined organic layers were washed with water to neutral pH, dried over sodium sulfate, and evaporated. A mixture of products was obtained as a yellowish oil, which was further purified by column chromatography on silica gel (hexane/ethyl acetate 4:6) to afford a yellow solid (43 mg, 39%). ^{1}HNMR (400 MHz, CDCl$_3$) δ 7.06 (2H, d), 6.66 (2H, d), 5.91 (1H, s), 4.50 (1H, s), [4.35 (s), 4.34 (s), (1H)], 2.91 (6H, s), 1.89 (3H, s), 0.56 (3H, s); MS *m/z* 446 (MH$^+$). [Spectra data match the published literature values.[85]]

DETERMINATION OF THE QUANTUM EFFICIENCY FOR SINGLE PHOTON EXCITATION.

To start a photolysis experiment, the UV absorption spectrum of the compound to be photolyzed is recorded. Based on the spectrum, an appropriate light source (similar wavelength as absorption peak) and vessel are selected. Most molecules have a rather broad absorption spectrum, often with several different absorption maxima. Usually, it is more efficient to select a light source with maximum emission matching one absorption maximum, and it is usually advantageous to select the maximum at the longest possible wavelength. Since the energy of the photons decreases as the wavelength increases, the severity of the side reactions may be decreased with longer wavelength light. For two-photon photolysis experiment, successful wavelength used in one-photon photolysis experiment was doubled.

The quantum yield for any process is the fraction of the photons used in the process, that is, the number of moles of compound undergoing the process divided by the number of moles of photons absorbed (1 mol of photons = 1 einstein). Therefore, the sum of the quantum yields for all the processes in which the photons have participated is equal to 1.

The energy of the light beam can be measured with a calibrated thermopile. Because it is extremely difficult to perform this type of absolute measurement routinely, indirect methods are usually preferred. They use as reference a reaction for which the quantum yield has been previous been measured. By determining the extent of product formation for the known reaction under conditions absolutely identical to those used for the unknown conversion, it is possible to determine how many quanta of light have been used. The chemical yield of the product formation in the unknown reaction is measured, and divided by the number of quanta to arrive at the yield of product for each quantum of light absorbed, the quantum yield. The reference

photochemical reaction mentioned above uses a starting material that is called an actinometer. In order to use an actinometer properly one must be certain that the quantum yield of the reference reaction and the reaction of interest are measured at the exact same wavelength(s). In this regard, the best actinometer is probably ferric oxalate.[83] Upon irradiation, the ferric ion is converted into Fe^{2+}, along with oxalate oxidation. The extent of conversion is determined by adding 1,10-phenanthroline complexes. The quantum yield has been carefully measured by Parker and Hatchard and found to vary little over a wide range of wavelengths.

UV absorption spectra of KMOPS-buffered solutions (3 mL) of the substrates (100 μM) in quartz cuvettes (21-Q-10, Starna, Atascadero, CA) were measured. Molar extinction coefficients were calculated. Then the test photolysis experiments were carried out by monitoring the UV absorption at 30 s, 1min, 2min, 3min, 4 min and 5min. The rough time needed for photolysis was estimated. Another 3 mL of KMOPS-buffered Bhc-diol caged aldehyde and ketone solutions were irradiated with 365-nm UV light from a mercury lamp (Spectroline SB-100P; Spectronics Corporation, Westbury, NY). The spectral output of the lamp is a distribution across the UV-A wavelengths (310-400 nm) with an intense band at 365 nm. A 20 μl aliquot of the solution was removed for analysis by HPLC at the point 0 s, 5 s, 10 s, 15 s, 20 s, 30 s, 40 s, 50 s, 60 s, 90 s, 120 s, and 240 s using an external standard method to determine concentrations. The compound was eluted with an isocratic mixture of acetonitrile and water containing 0.1% trifluoroacetic acid (flow rate of 1 mL/min). The exact HPLC conditions are shown in Table 2. The progress curves were plotted as simple decaying exponentials. Quantum efficiencies (Q_{u1}) were calculated according to a published method,[35] using $Q_{u1} = (I \cdot \sigma \cdot t_{90\%})^{-1}$, where I is the irradiation intensity in $ein \cdot cm^{-2} \cdot s^{-1}$, σ is the decadic extinction coefficient (10^3 times ε, molar extinction coefficient) in $cm^2 \cdot mol^{-1}$, and $t_{90\%}$ is the irradiation time in seconds for

90% conversion to product. The UV intensity of the lamp I was measured by using potassium ferrioxalate actinometry[83] in the same setup. 3 mL of 6 mM potassium ferrioxalate solution were irradiated for 20 s and mixed properly. 2 mL of this solution was mixed with 3 mL aqueous buffer (600 mL1 M NaOAc and 360 mL 1N H_2SO_4 diluted to 1 L with water), 3 mL of 0.1% 1,10-phenanthroline solution (10 mg in 100 mL water), and 1 mL of 2 M KF solution and the mixture was diluted with water to 25 mL in a volumetric flask. A blank solution was prepared without irradiation in the same way. These solutions were allowed to stand for 30 min in the dark. The UV absorption of these solutions was measured at 510 nm and the UV lamp intensity I was calculated by the following equation.

$$I = \frac{V_3 \Delta D_{510}}{10^3 \varepsilon_{510} V_2 \phi_{Fe^{2+}} t}$$

V_3 is the volume of dilution (25 mL); V_2 is the volume of irradiated solution taken for analysis; ΔD_{510} is the absorption of the absorption of the solution; ε_{510} is the molar extinction coefficient of actinometry; $\phi_{Fe^{2+}}$ is the quantum yields for production of ferrous ions from potassium ferrioxalate at about 365 nm (1.21); and t is the time of irradiation. Release of piperonal or acetophenone was monitored at 313 and 240 nm, respectively, and the progress curves plotted as an exponential rise to max. Concentrations were determined using an external standard by making a calibration curve for each compound detected.

Table 2. HPLC Conditions of Single- and Two-photon Photolysis

Bhc-diol protected aldehydes and ketones	Solvent conditions for HPLC (volume percentage)	Compounds were monitored	Detection wavelengths (nm)	Retention time (min)
Bhc-diol-benzaldehyde	Acetonitrile 65%; H_2O (0.1% TFA) 35%	Bhc-diol-benzaldehyde	325	5.533
Bhc-diol-piperonal	Acetonitrile 60%; H_2O (0.1% TFA) 40%	Bhc-diol-piperonal	329	5.944
		Piperonal	313	4.234
Bhc-diol-cyclohexanone	Acetonitrile 68%; H_2O (0.1% TFA) 32%	Bhc-diol-cyclohexanone	328	5.910
Bhc-diol-acetophenone	Acetonitrile 82%; H_2O (0.1% TFA) 18%	Bhc-diol-acetophenone	328	3.832
		Acetophenone	240	3.454

DETERMINATION OF THE DARK HYDROLYSIS RATE.

Substrates were dissolved in a KMOPS-buffered solution and stored in the dark at room temperature. HPLC analysis was carried out periodically as described for single photon photolysis.

CONTROL PHOTLOLYSIS EXPERIMENTS.

Piperonal (97 μM) and acetophenone (104 μM) were each dissolved in KMOPS buffer. A small amount of MeOH was used as a co-solvent to effect dissolution. The solutions were

exposed to the 365-nm mercury lamp for 1 min, and any change in concentration was monitored by HPLC as in the quantum efficiency determination. KMOPS-buffered solutions of Bhc-diol (100 μM) and carbonyl compound (97 μM, 104 μM) were photolyzed as before, and any changes in concentration of Bhc-diol, piperonal, or acetophenone were monitored by HPLC.

MEASUREMENT OF THE TWO-PHOTON UNCAGING CROSS-SECTION.

Measurements were carried out in microcuvettes (10×1×1 mm illuminated dimensions) with an effective filling volume of 20 μl (26.10F-Q-10, Starna, Atascadero, CA) using light from a fs-pulsed and mode-locked Ti:Sapphire laser (Mira 900 pumped by a Verdi, Coherent, Santa Clara, CA) focused on the center of the cuvette chamber with a 25 mm focal length lens optimized for IR lasers (06LXP003/076, Melles-Griot, Irvine, CA). The two-photon uncaging cross-section (δ_{u2}) was estimated by referencing to fluorescein, a compound with a known two-photon fluorescence quantum yield (Q_{f2} = 0.9 mol/ein) and absorbance cross-section (δ_{aF} = 30 GM at 740 nm).[47] A fluorescein solution was prepared: 10 μL of 1 M NaOH in 10 mL of water, and 1 mg of fluorescein. Then 300 μL of this solution was dissolved in 3 mL of water. Its concentration was measured by UV spectrophotometry (ε = 88000 cm^{-1} M^{-1}). The fluorescein solution (20 μL) was put into the microcuvette and placed in the laser path. The fluorescence intensity was measured by a radiometer, both at the beginning and the end of the experiment. A series of 20-μl aliquots of the solution, containing the caged compound, were placed in the cuvette chamber and irradiated three times at each exposure time: 0, 5, 10, 20, 30, and 40 min. Each aliquot was analyzed for the concentration of the caged compounds. The same HPLC conditions were used as in the one-photon photolysis experiment. The progress curves were plotted as simple decaying exponentials. Release of piperonal or acetophenone was monitored at

313 and 240 nm, respectively, and the progress curves plotted as an exponential rise to max. Concentrations were determined using an external standard by making a calibration curve for each compound detected.

The two-photon uncaging cross-sections were calculated according to the following equation:

$$\delta_u = \frac{N_p \phi Q_{f2} \delta_{aF} C_F}{\langle F(t) \rangle C_s}$$

Where N_p is the number of product molecules formed per unit time (molecules/s, determined by HPLC analysis as in the single photon analysis); ϕ is the collection efficiency of the detector (SED033 on an IL-1700, International Light, Newburyport, MA) used to measure the fluorescence of fluorescein emitted at a right angle to the beam and passed though a 535/45 nm bandpass filter (Chroma Technologies, Brattleboro, VT); C_F is the concentration of fluorescein (mol/L); $<F(t)>$ is the time averaged fluorescent photon flux (photons/s) collected by the detector; and C_s is the initial concentration of caged substrate (mol/L).[35]

The collection efficiency of the detector ϕ was calculated by the following equation:

$$\phi = \frac{Ay}{4\pi R^2 n^2}$$

A: area of detector (0.38 cm^2)
y: fraction of integrated emission spectrum transmitted by interference filter (0.465)
R: distance from the center of the cuvette to the detector (2.25 cm)
n: refractive index of water (1.333)

The number of product molecules formed per unit time N_p was calculated by the following equation:

$$N_p = \frac{C_s V_s A' - H C_s V_s A'}{t}$$

C_s: concentration of substrate
V_s: volume of substrate
A': 6.022×10^{23} molecules/mole
H: fraction of substrate remaining
t: time (s)

The time averaged fluorescent photon flux (photons/s) collected by the detector $<F(t)>$ was calculated by the following equation:

$$<F(t)> = \frac{FA\lambda}{rhc}$$

F: fluorescence reading (A)
A: area of detector (0.38 cm^2)
λ: wavelength (535×10^{-9} m)
r: spectral response of detector (0.09385 at 535 nm)
h: Plank's constant (6.63×10^{-34} J $\cdot$ s)
c: speed of light (3.00×10^{8} m/s)

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